Protein recovery

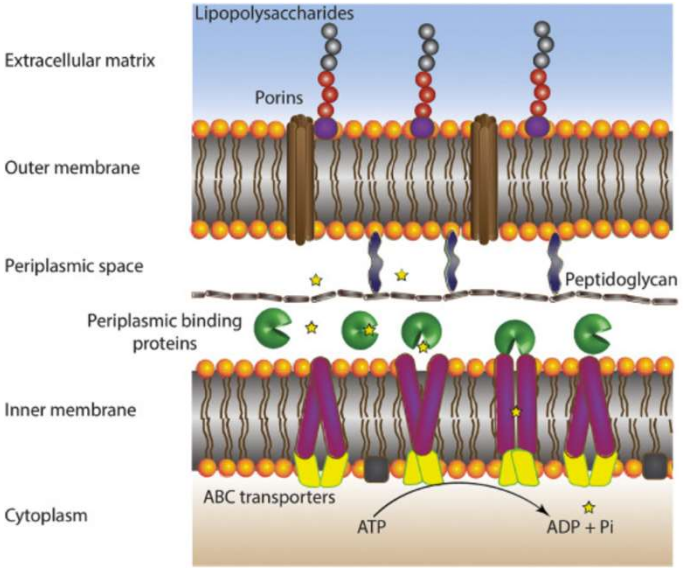
- Cytosol expressed protein: cell lysis

Enzymatic, mechanical, ultrasounds, chemical.

- Periplasm expressed protein: mild lysis

EDTA, osmotic shock, detergents

- Secreted protein: media recovery



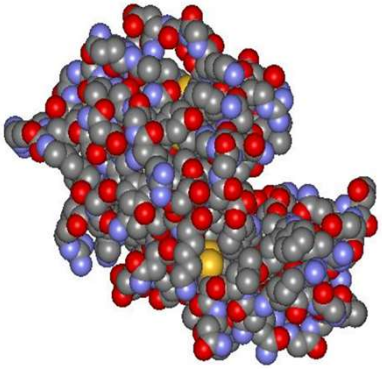
Mammalian and insect cells: relatively easy to break, use of hypotonic solution e/o detergent (Tween20 e Triton).

Bacteria and yeast: harder to disrupt, especially yeast. Combination of multiple techniques.

Cell disruption

Enzymatic method: **Lysozyme** for bacteria hosts; **zymolyase** for yeast

Ultrasound method: **Sonication** creates localized high pressure breaking cell membranes



Mechanical method: **Bead mill** disrupt with beads and high frequency agitation; **French press & Microfluidizer** disrupt forcing sample trough a narrow tube/valve under high pressure

Bead mill

French press & Microfluidizer

Chromatographic methods

Tips to design optimal purification strategy:

1) Be aware of contaminants at each step of purification

2) Choose detection method (usually UV A_{280nm}, but also fluorescence, conductivity, light scattering, …)

3) Choose purity assessment method (SDS-PAGE, WB, MassSpec…)

4) Check stability of protein: pH, temperature, detergents, ionic strength, additives, organic solvents; sensitivity to proteases

5) Tags ⟶ Affinity chromatography

6) pI ⟶ Ion EXchange chromatography

} Concentrate protein

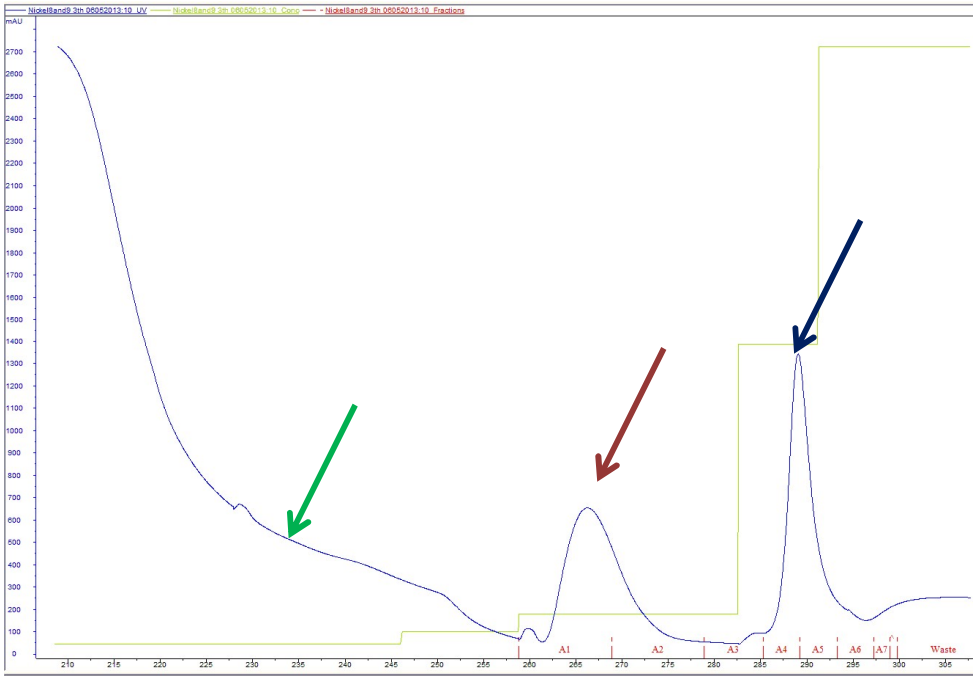
7) MW ⟶ Gel Filtration chromatography

Affinity chromatography

affinity of proteins for specific chemical groups: (ATP, DNA, immunoglobulin fold).

- His-tag
- GST-tag
- MBP-tag

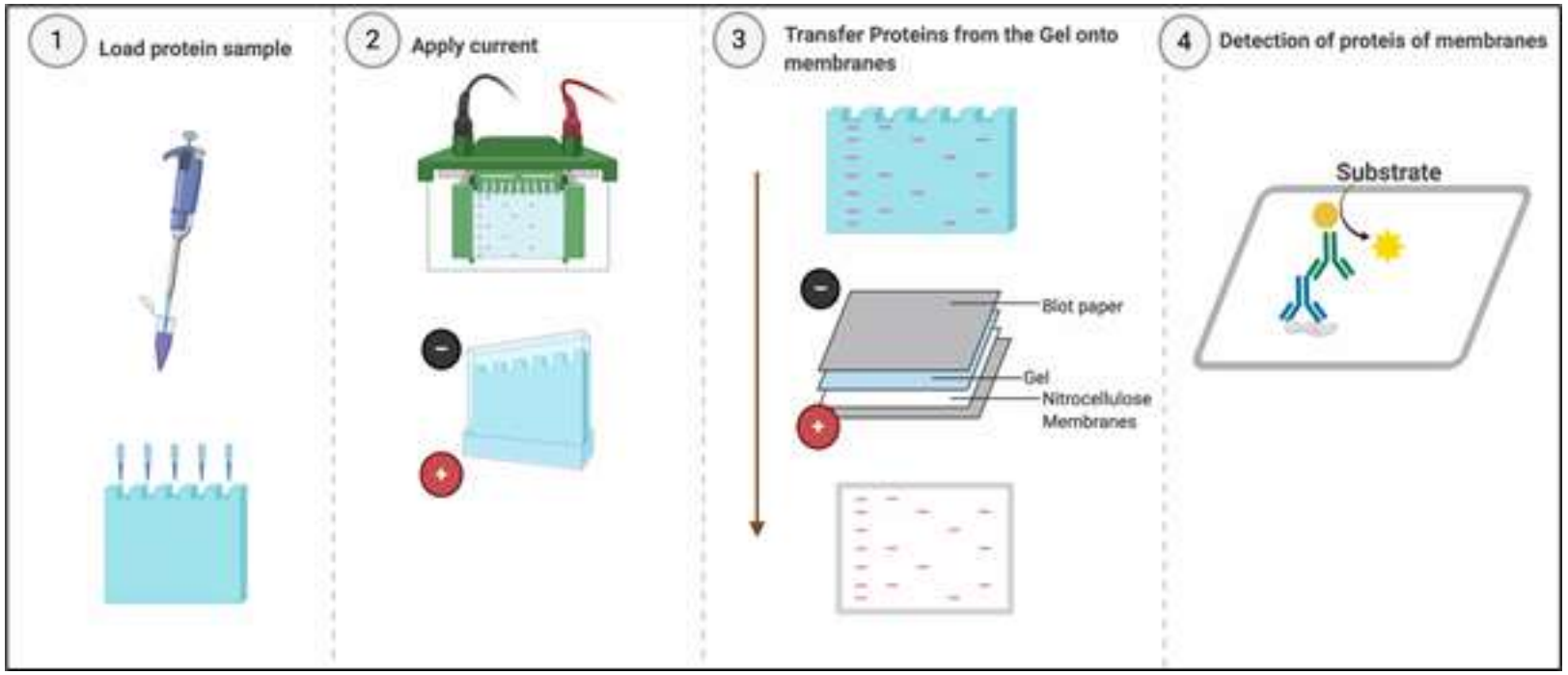
- Transition metal ion (Ni^{2+}, Co^{2+},)
- Glutathione
- Amylose



- Loading
- Wash 1 (unbound proteins)
- Wash 2 (loosely bound protein)
- Elution with a competitor (protein of interest)

Detection method

Separation of proteins through an electric field. **Proteins** are **unfolded** and covered by **SDS**, an anionic detergent. Mobility of **proteins** depends on their dimension (≈ **molecular weight**)



<u>Protein detection:</u>

Coomassie Blue staining

Silver staining

Western blotting

<u>Western Blot/dot blot:</u>

Transfert on membrane

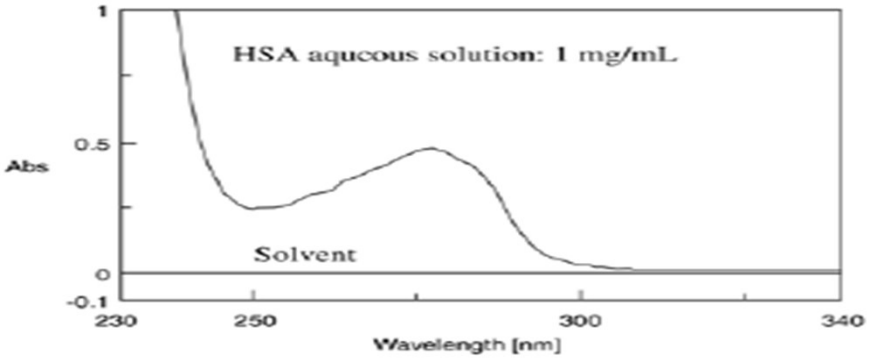
Primary antibody + Secondary antibody

Detection

Concentration

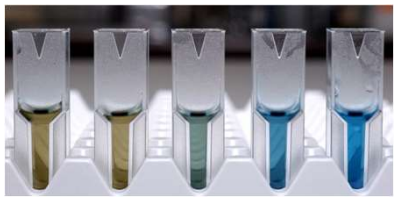
UV-VIS Spectrometry

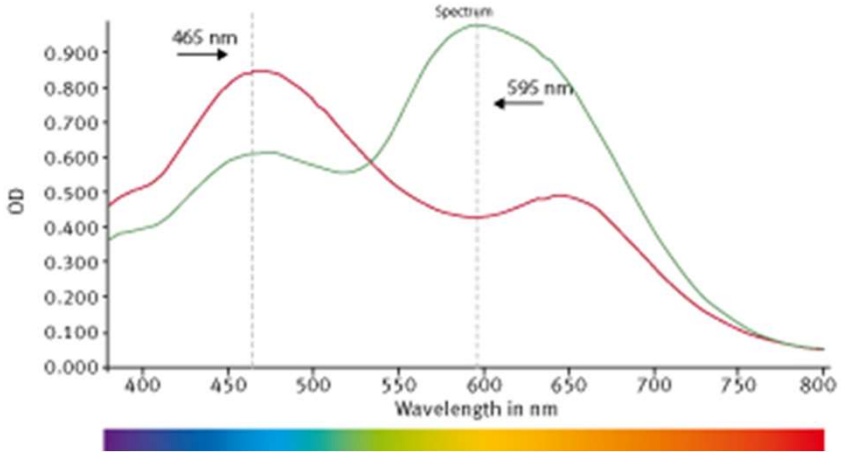
Proteins absorb **UV light** (max **275-280 nm**, Trp, Tys, Phe). Lambert-Beer's law: **Abs = ε · C · l**, ε calculated on the primary sequence



Bradford assay

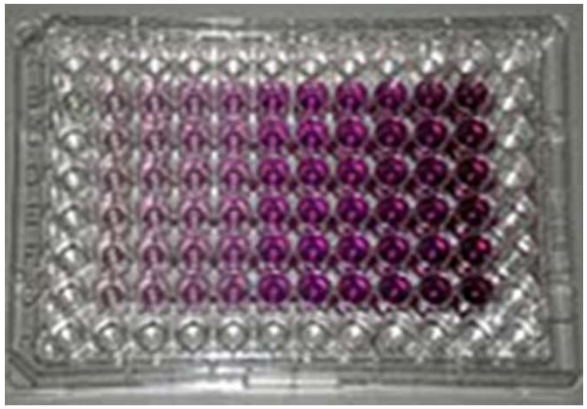
Coomassie Brilliant Blue G-250 binding the protein (depends on the protein sequence). Concentration can be evaluated using **a standard,** absorbance at **595 nm** is measured





BCA assay (Bicinchoninic Acid)

Cu2+ to protein sample: **peptide bond** is oxidized and **reduces Cu2+ to Cu+**: independent from protein, but depend on the protein mass!! Concentration can be evaluated using **a standard,** absorbance measurement at **562 nm**.



Ionic exchange chromatography (IEX)

allows the separation of ions and polar molecules based on the charge properties of the molecules.

- **cation exchange chromatography S/Heparin (resin** charge **-**, retention of **+ charged molecules)**

- **anion exchange chromatography Q/DEAE (resin** charge **+**, retention of **- charged molecules)**

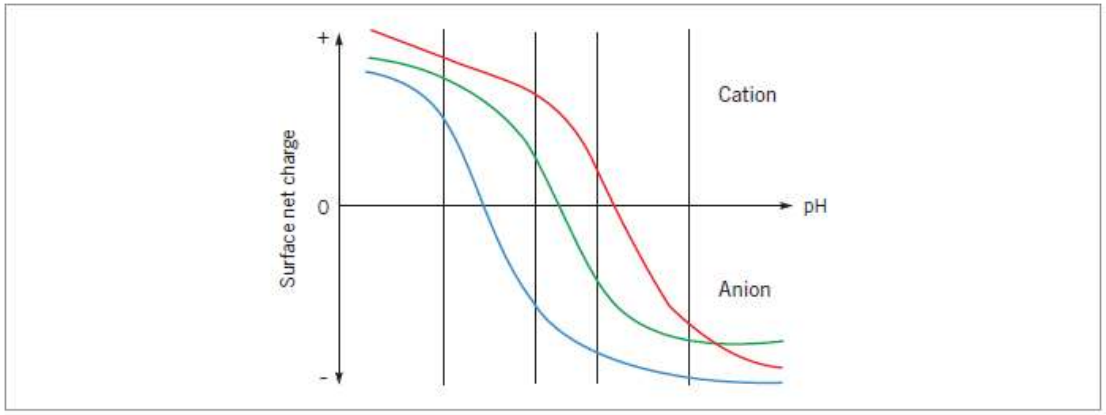
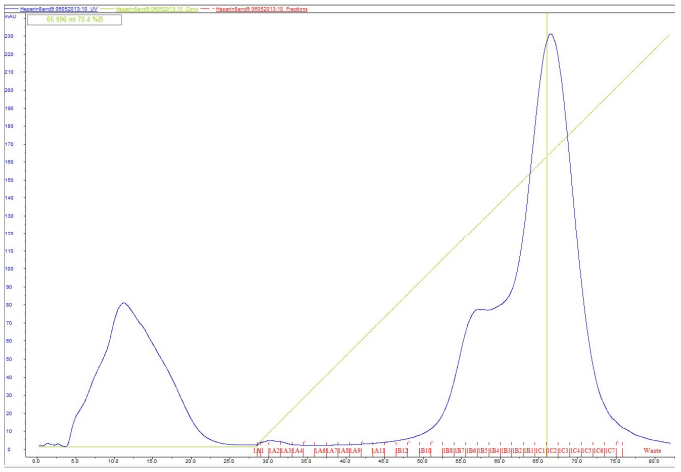


Fig. 2. Theoretical protein titration curves, showing how net surface charge varies with pH.

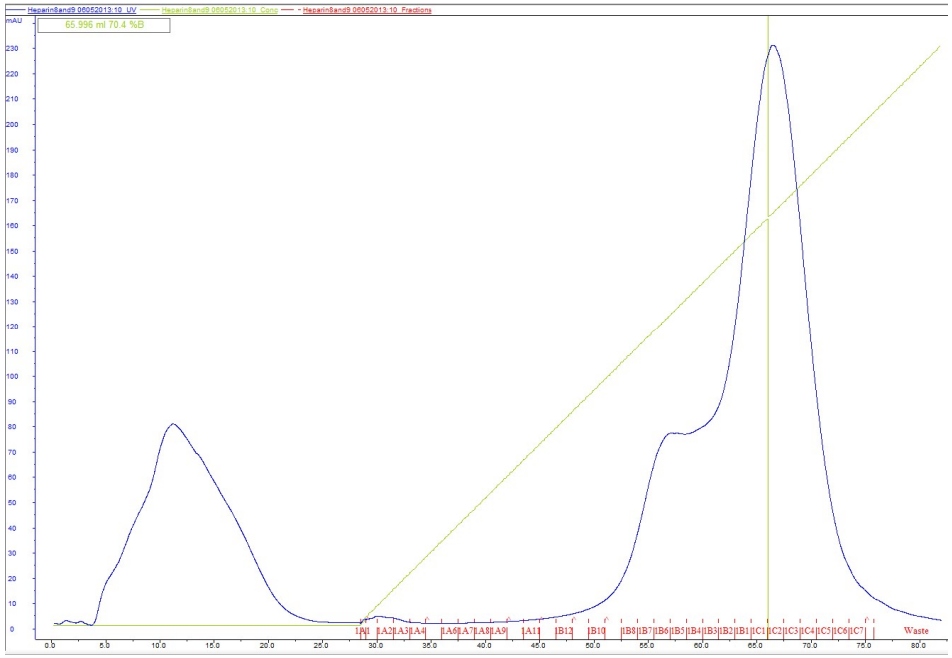


Buffer pH, beads side influences the separation, elution done with salt

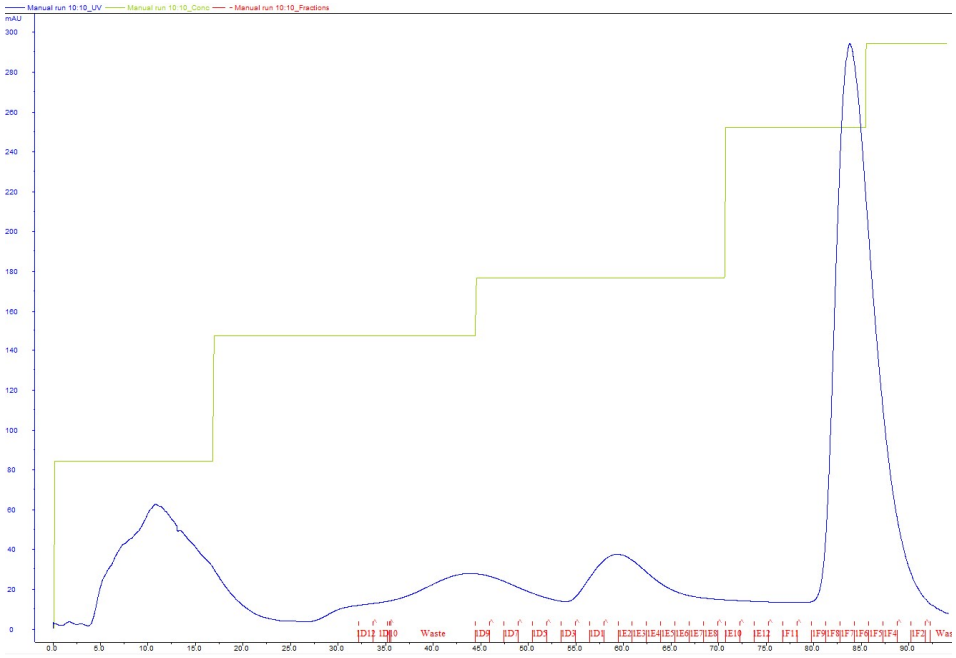
- Capture step (protein isolation)
- Intermediate step (remove impurities)
- Polishing step (remove traces of contaminants or misfolded proteins)

IEX

- Capture step (protein isolation)
- Intermediate step (remove impurities)
- Polishing step (remove traces of contaminants or misfolded proteins)



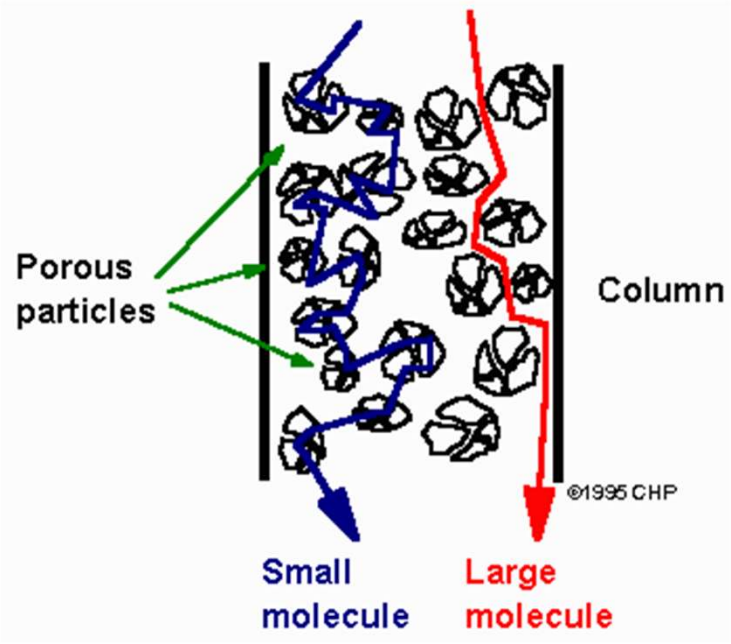
Linear gradient

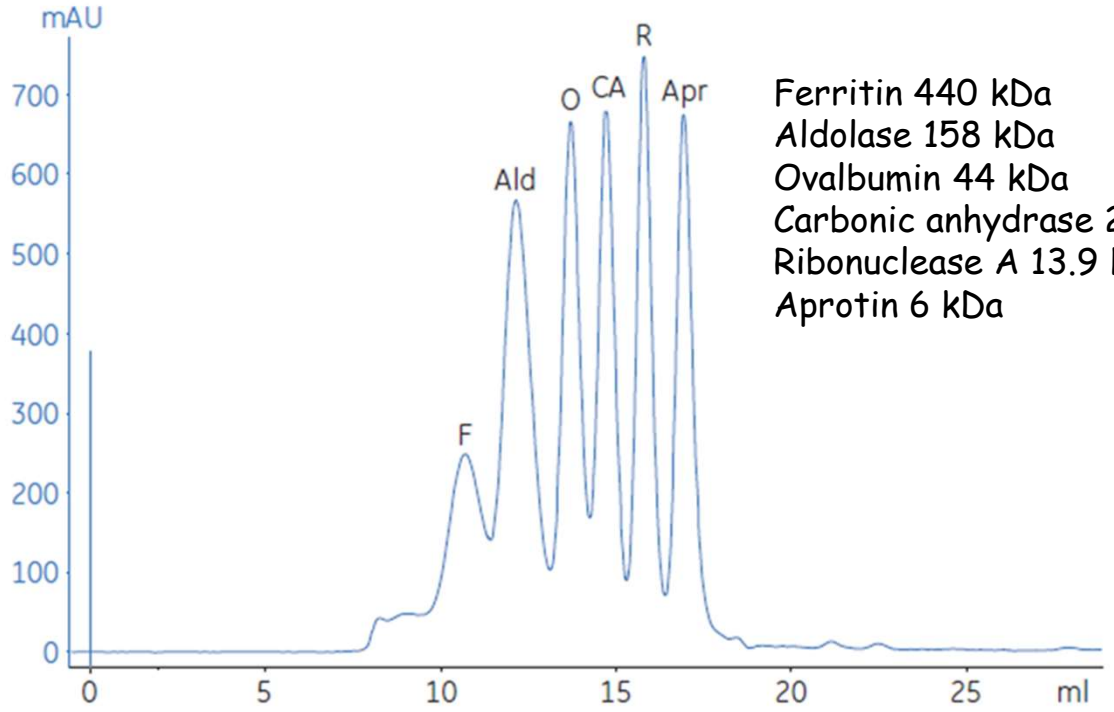


Step gradient

Size exclusion chromatography (SEC)

molecules in solution are separated by their size and molecular weight,
Big molecules make a short path, smaller molecules are eluted later

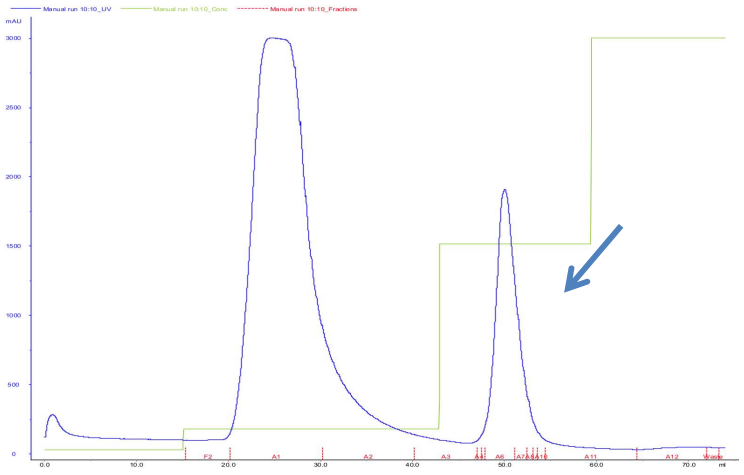




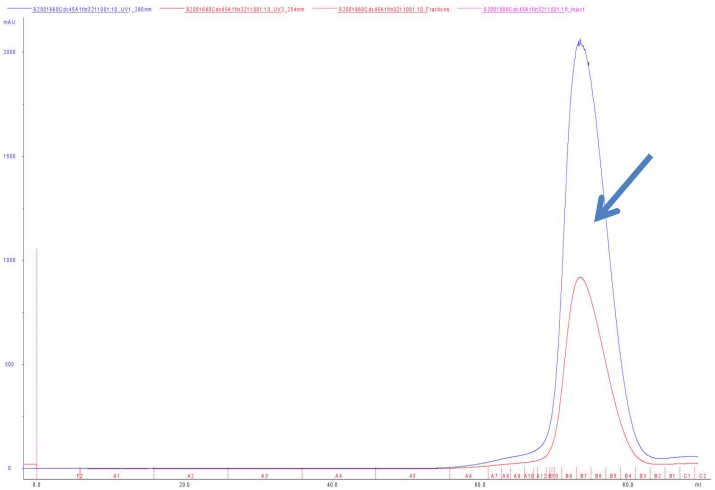
Ferritin 440 kDa
Aldolase 158 kDa
Ovalbumin 44 kDa
Carbonic anhydrase 29 kDa
Ribonuclease A 13.9 kDa
Aprotin 6 kDa

- Polishing step
- Oligomeric state separation
- Buffer exchange

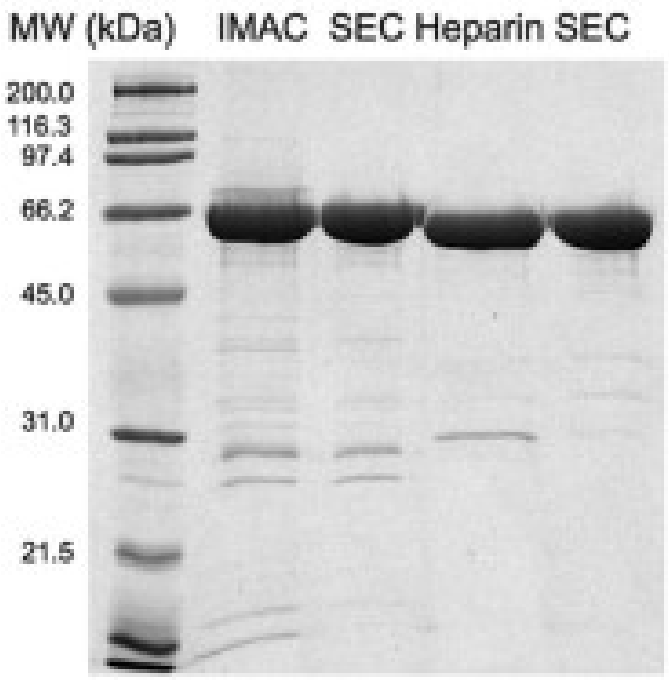
hCdc45 FL purification



IMAC- HiTrap Chelating HP 5 ml



GF-Superdex 200 16/60



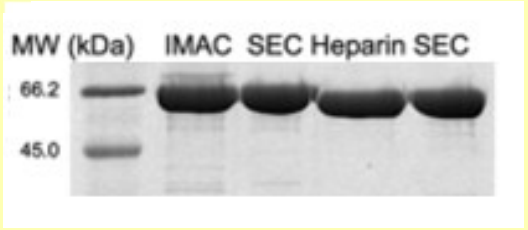
- IMAC (Ni-NTA affinity)
- SEC (Superdex 200)
- TEV Cleavage
- Heparin
- SEC (Superdex 200)

Protein characterization

Structural studies
- Crystallization,
- Saxs,
- EM
- NMR

Biochemical studies
- protein-protein interaction,
- substate specificity,
- analytical ultracentrifugation

Protein of interest



Quality control
- Mass Spec,
- Thermofluor assay
- N/C term sequencing

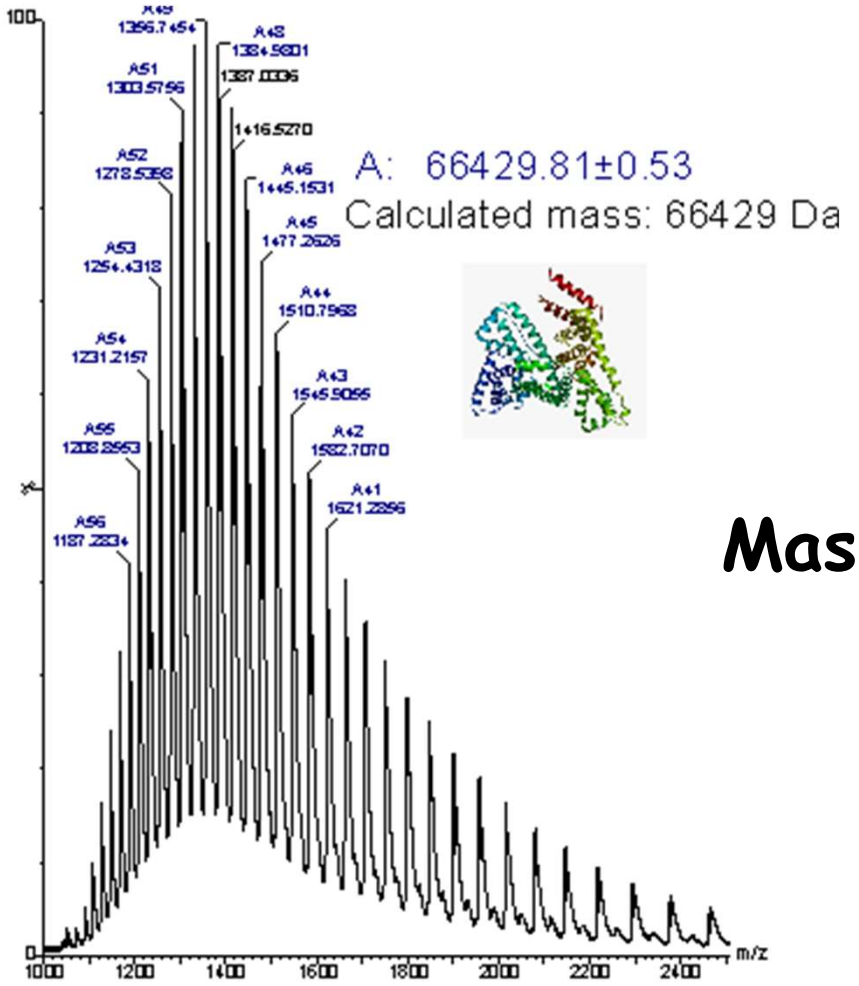
Biophisical studies
- Calorimetry,
- CD

Protein characterization

Protein parameter	Techniques
IDENTIFICATION & PURITY	(SDS-PAGE), Western Blot, Mass-spectrometry, Nterm-sequencing
CONCENTRATION	UV-VIS, Bradford assay, BCA assay
STABILITY	Thermo Stability Assay (TSA), Circular Dichroism (CD), Differential Scanning Calorimetry (DSC), UV-VIS
OLIGOMERIC STATE & AGGREGATION	SEC-Mals, Dynamic Light Scattering (DLS), Transmission Electron Microscopy (TEM), Sucrose Gradient Ultracentrifugation
CHEMICAL HETEROGENEITY	Mass Spectrometry, SDS-PAGE
CONFORMATIONAL HETEROGENEITY	TEM, NMR
FOLDING	TSA,CD, DSC,, Spectrofluorimetry, Deutorium Exchange Mass Spectrometry (DXMS)
ACTIVITY, LIGAND BINDING	Biochemical assays, Surface Plasmon Resonance (SPR), Isothermal Titration Calorimetry (ITC) microscale thermoforesis (MST)

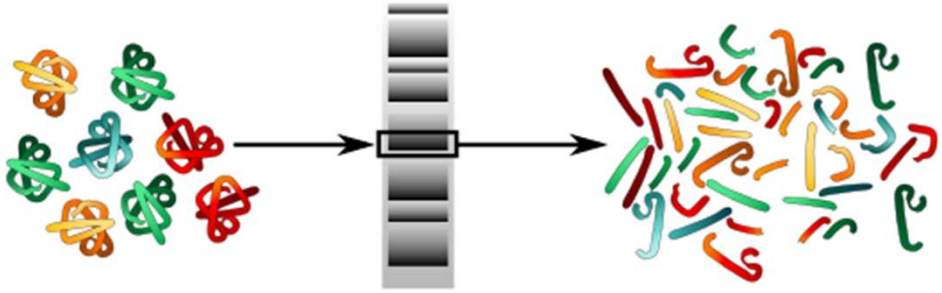
Chemical heterogeneity: Mass Spectrometry (MS)

the protein integrity, PTM detection



Mild ionization methods (ESI or MALDI), charging of the protein, deconvolution of the spectrum allows determination of exact mass of the protein

MassSpec after enzymatic digestion:

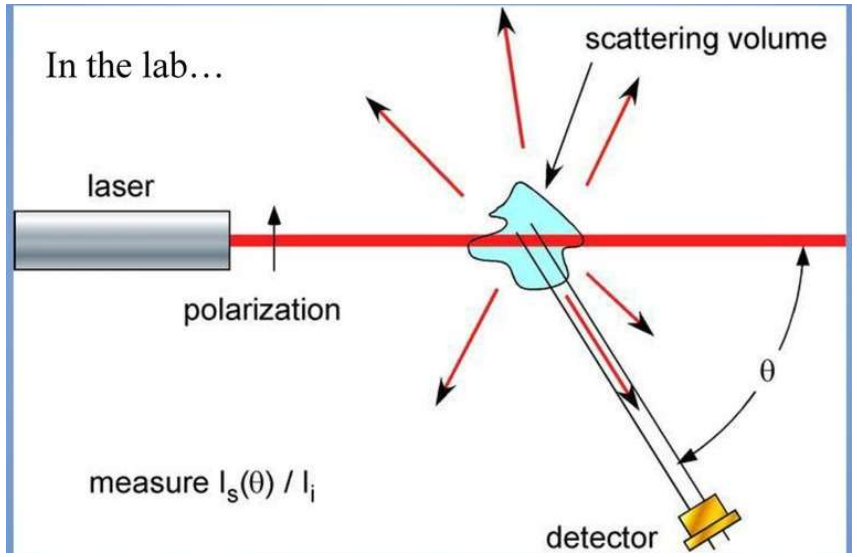


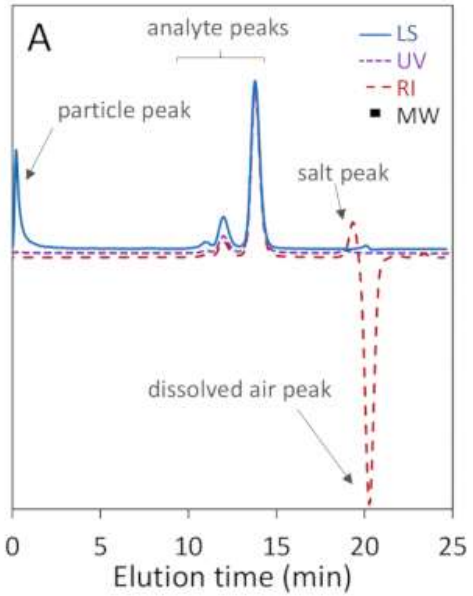
Liquid Chomatography-MS ⟵ Database of protein: contaminant identification, domain identification, PTM fragment

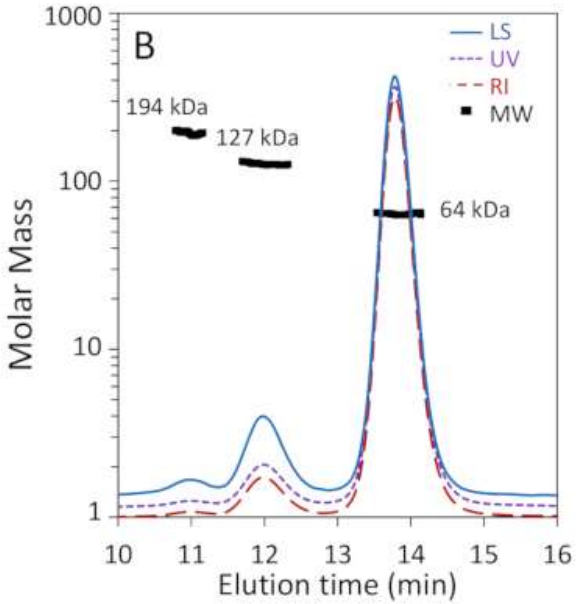
SEC-Mals olicomeric state

the combination of size-exclusion chromatography with multi-angle light scattering

A MALS detector measures the proportion of light scattered by an analyte into multiple angles relative to the incident laser beam, instrumentation determines MW independently of elution time

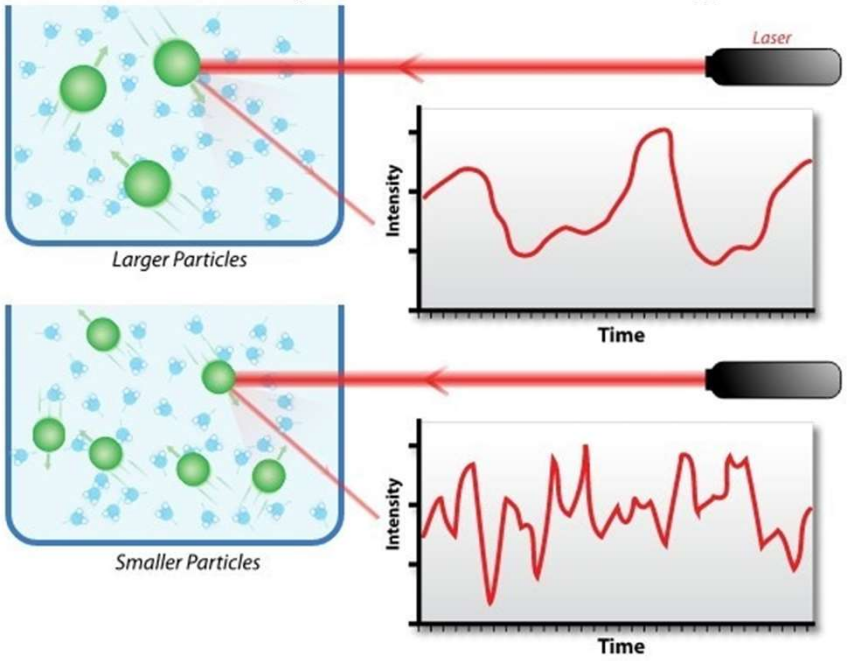






$$I(\theta)_{\text{scattered}} \propto Mc\left(\frac{dn}{dc}\right)^2 P(\theta)$$

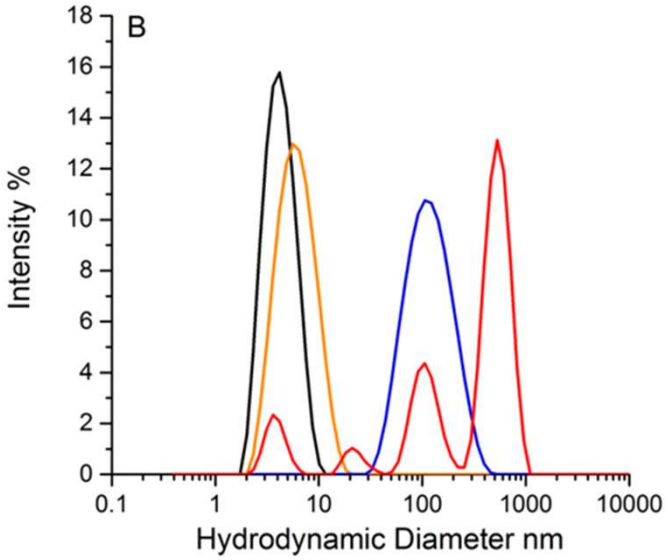
Oligomeric state:
Dynamic Light Scattering (DLS)



Measurement of fluctuations of scattered light in time.

Autocorrelation function gives the diffusion coefficient of particles in solution.

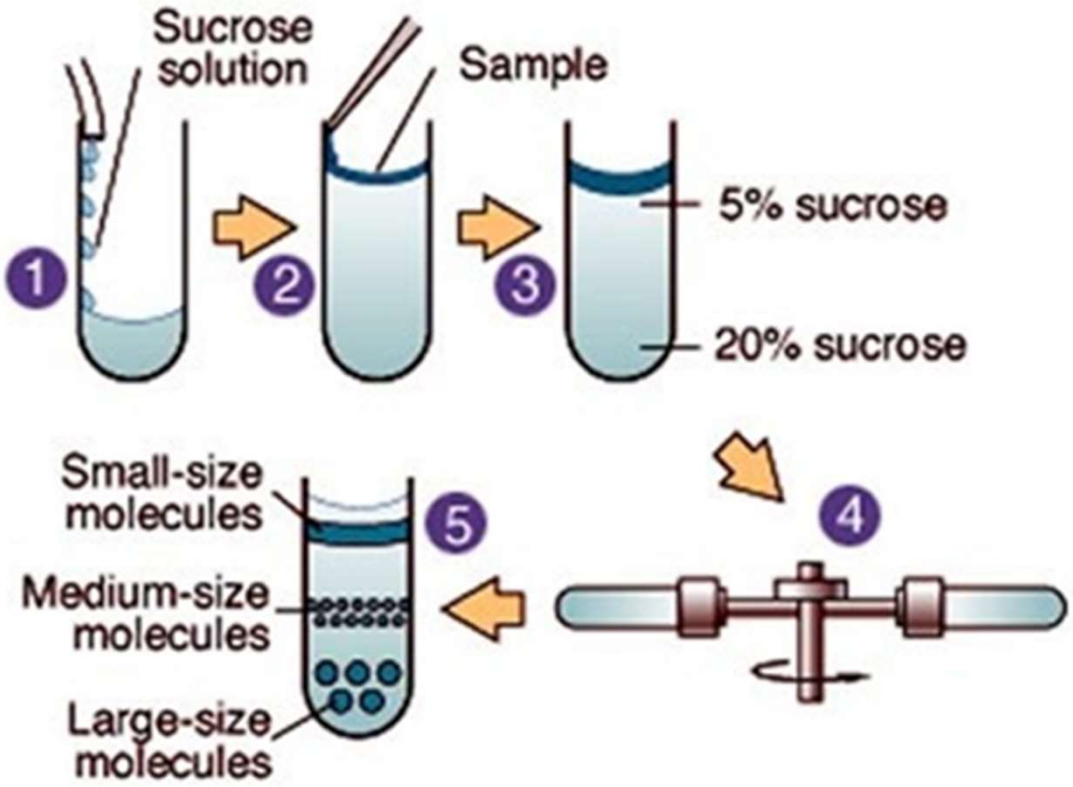
Known temperature and viscosity Stokes-Einstein equation allows to hydrodynamic radius of the particle.



Used to determine:

- Dimension of a protein (also combined with SEC)
- Aggregation state
- Oligomerization state
- Interactions between proteins

Oligomeric state:
Sucrose Gradient Ultracentrifugation



Separates particles by **density**:

protein density = the sucrose density

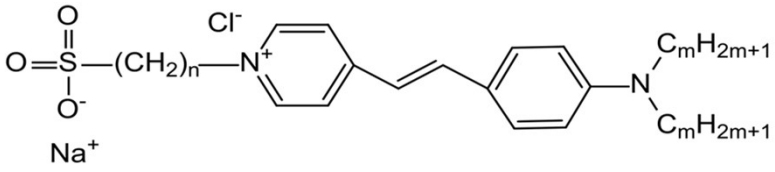
> 150.000 *g*

Used to determine:

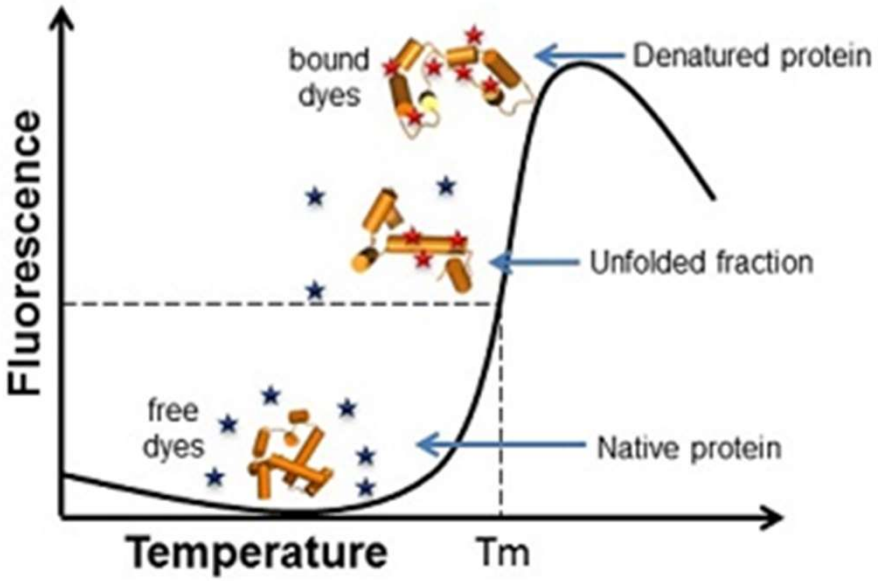
- Molecular Weight (MW)
- Oligomerization state
- Protein-protein interactions
- Shape of the protein

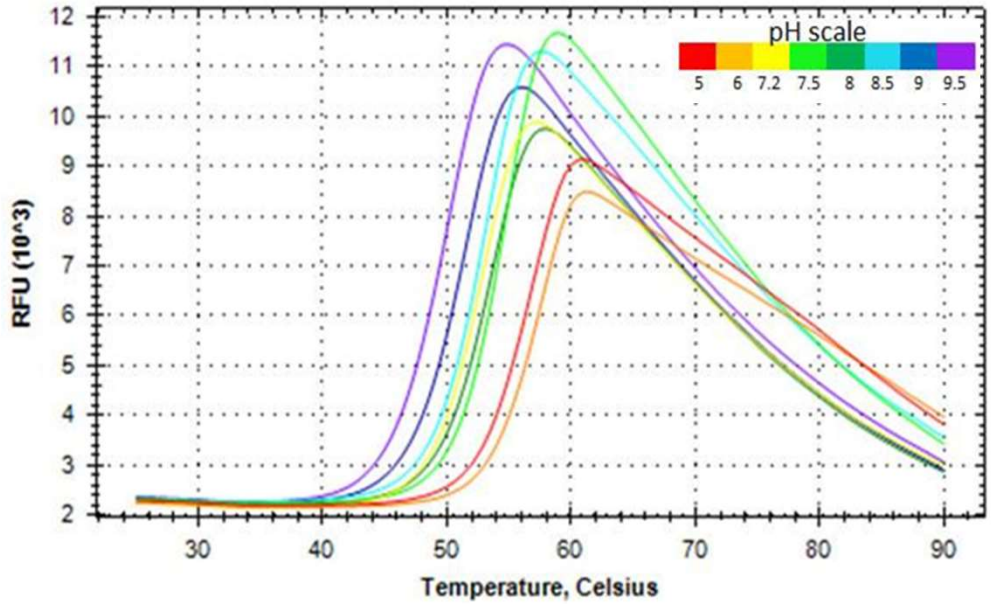
Stability: Fluorescent Thermal Shift Assay (TSA)

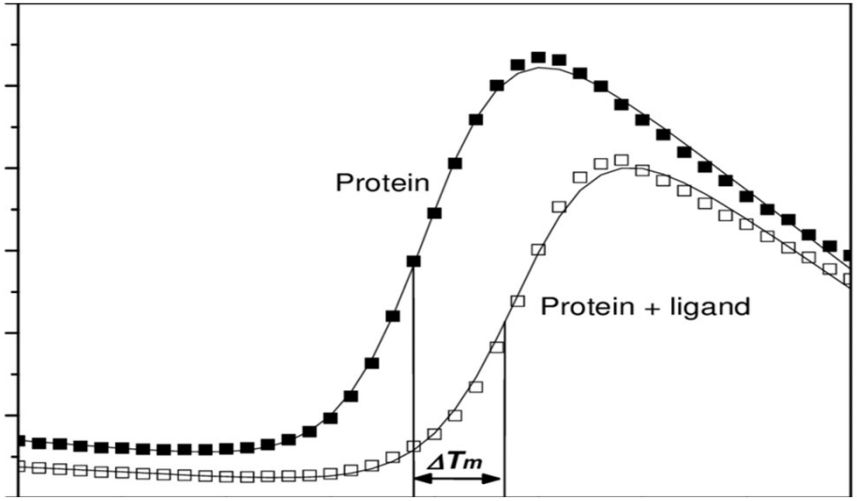
Sypro Orange dye: fluorescence only when bound to the hydrophobic surfaces of the protein. Fluorescence is quenched by water.



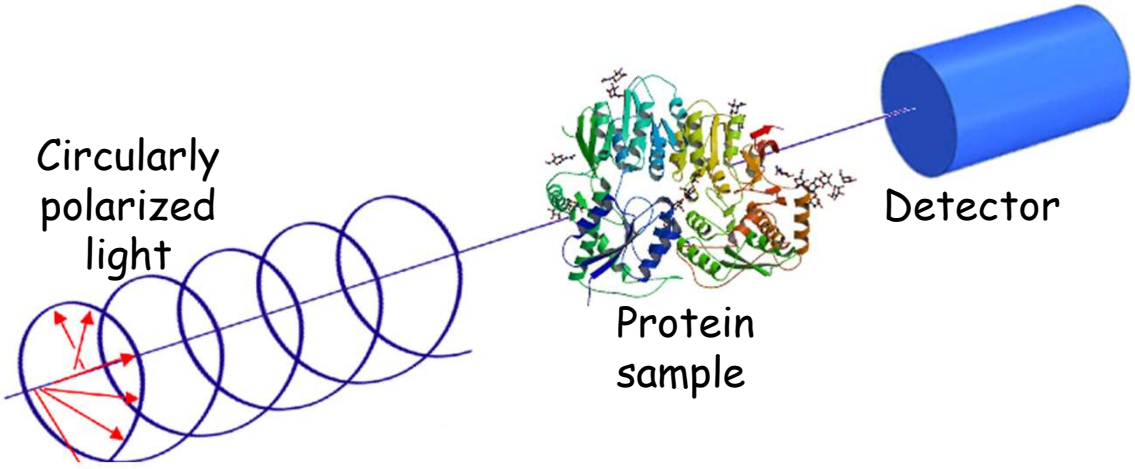
- **Protein stability**: fluorescence vs temperature
- **Optimization of protein buffer**
- **Analysis of ligand binding**: comparing melting curves of *apoprotein* and protein/ligand



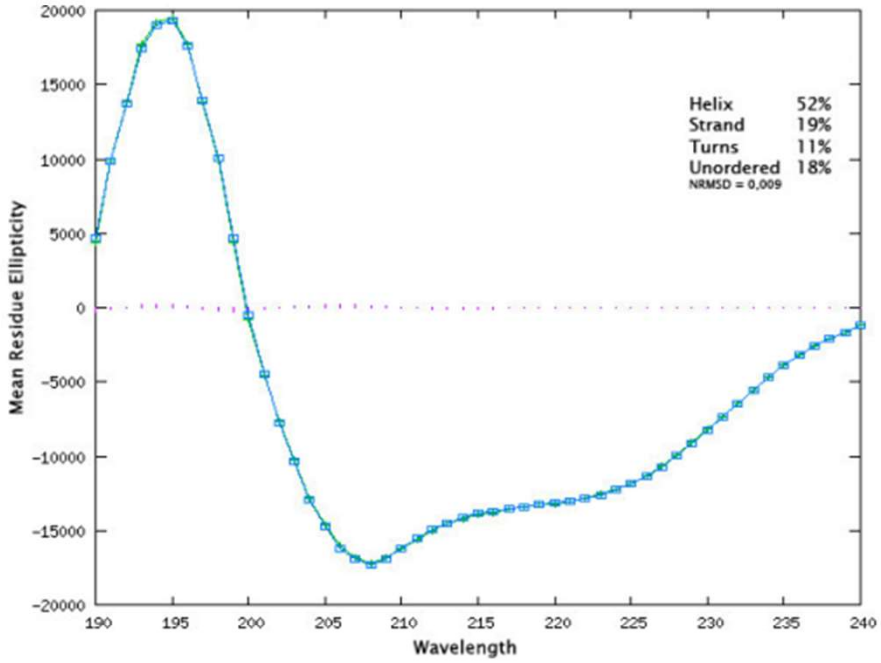


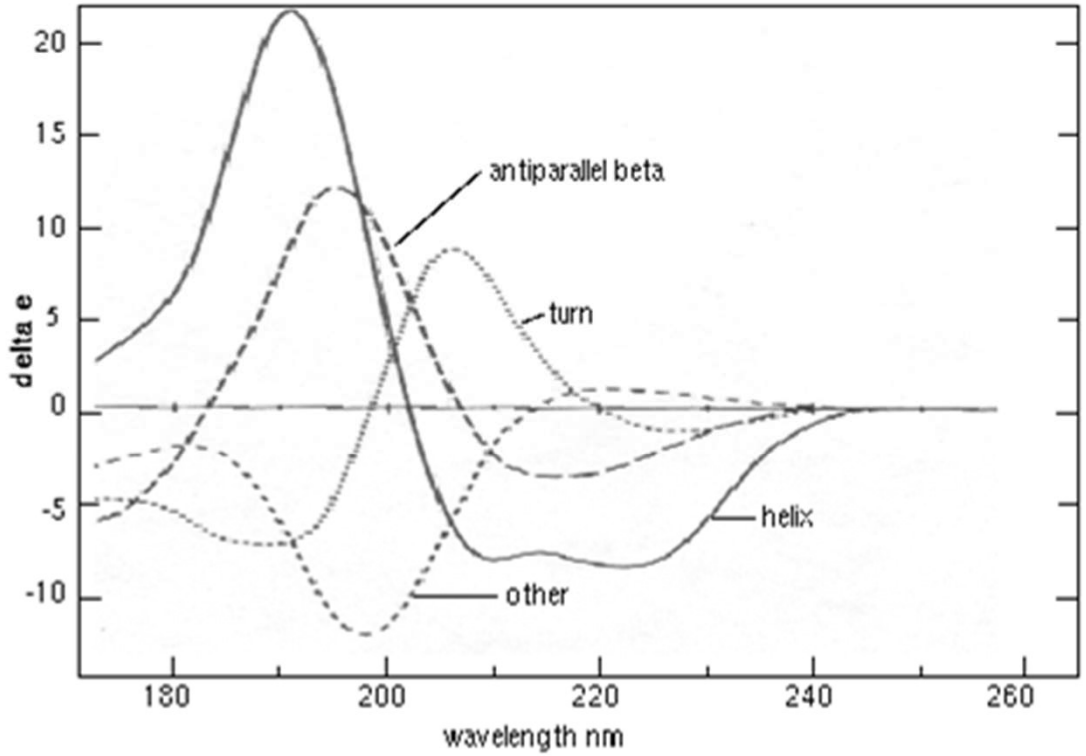


Stability, Protein folding: Circular Dichroism (CD)



Absorption of Left and Right Circularly Polarized (LCP and RCP) light is measured.
Ellipticity measured difference absorption of LCP and RCP.
Ellipticity is plot against wavelength.

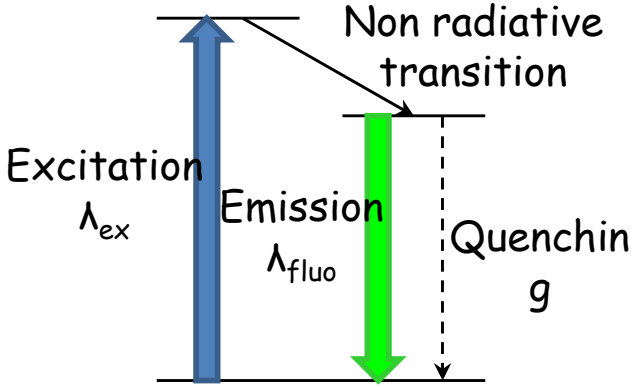




CD spectrum gives information on:

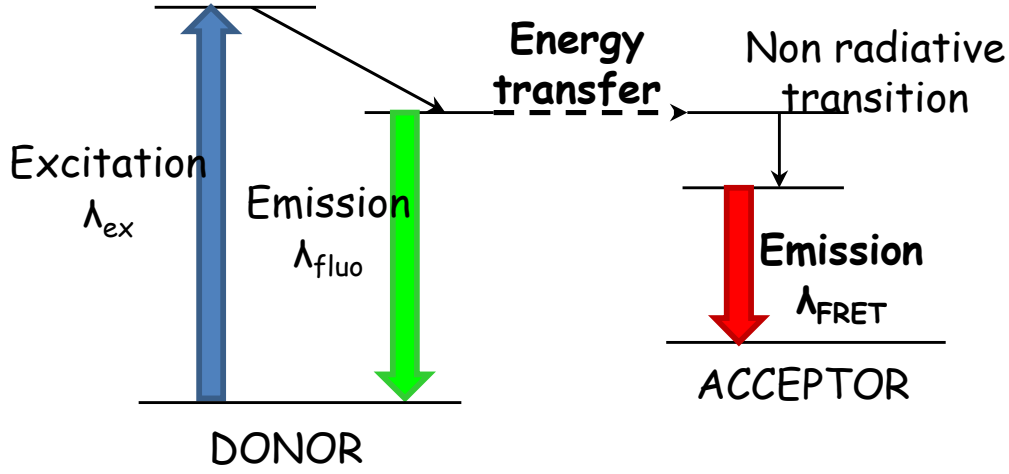
- Folding of protein
- **Stability & melting temperature**
- Conformational changes

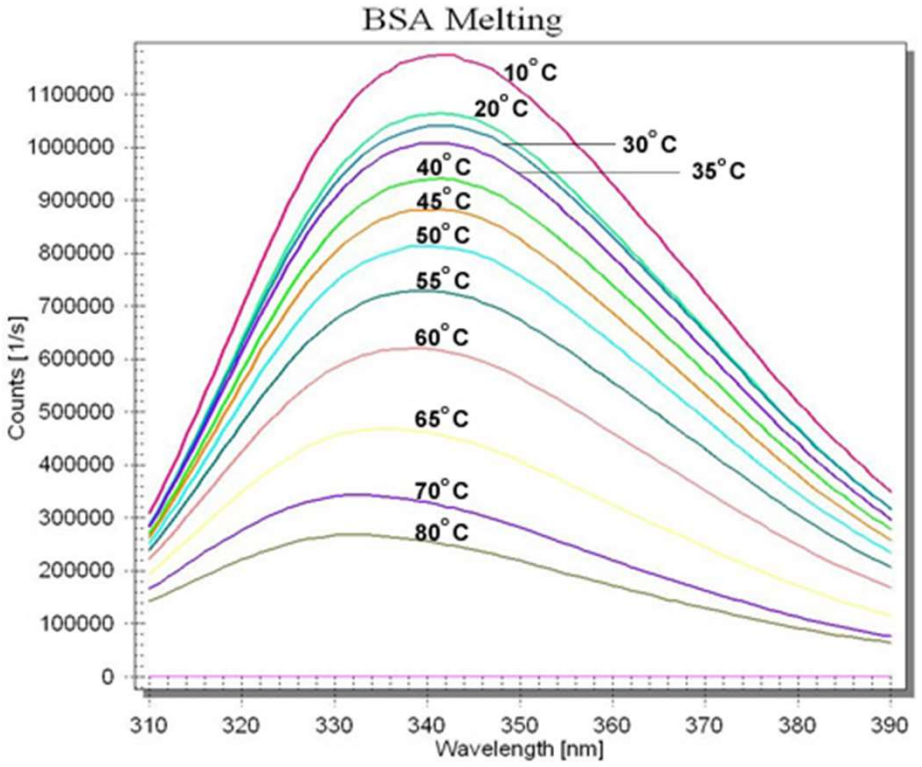
Ligand binding: Spectrofluorimetry and/or Fluorescence Resonance Energy Transfer (FRET)



Excitation at λ_{ex} Emission at λ_{fluo} ($> \lambda_{ex}$)

Aromatic residues display fluorescence or
Using of fluorescent probes that increase/quench their fluorescence due to interaction with the protein

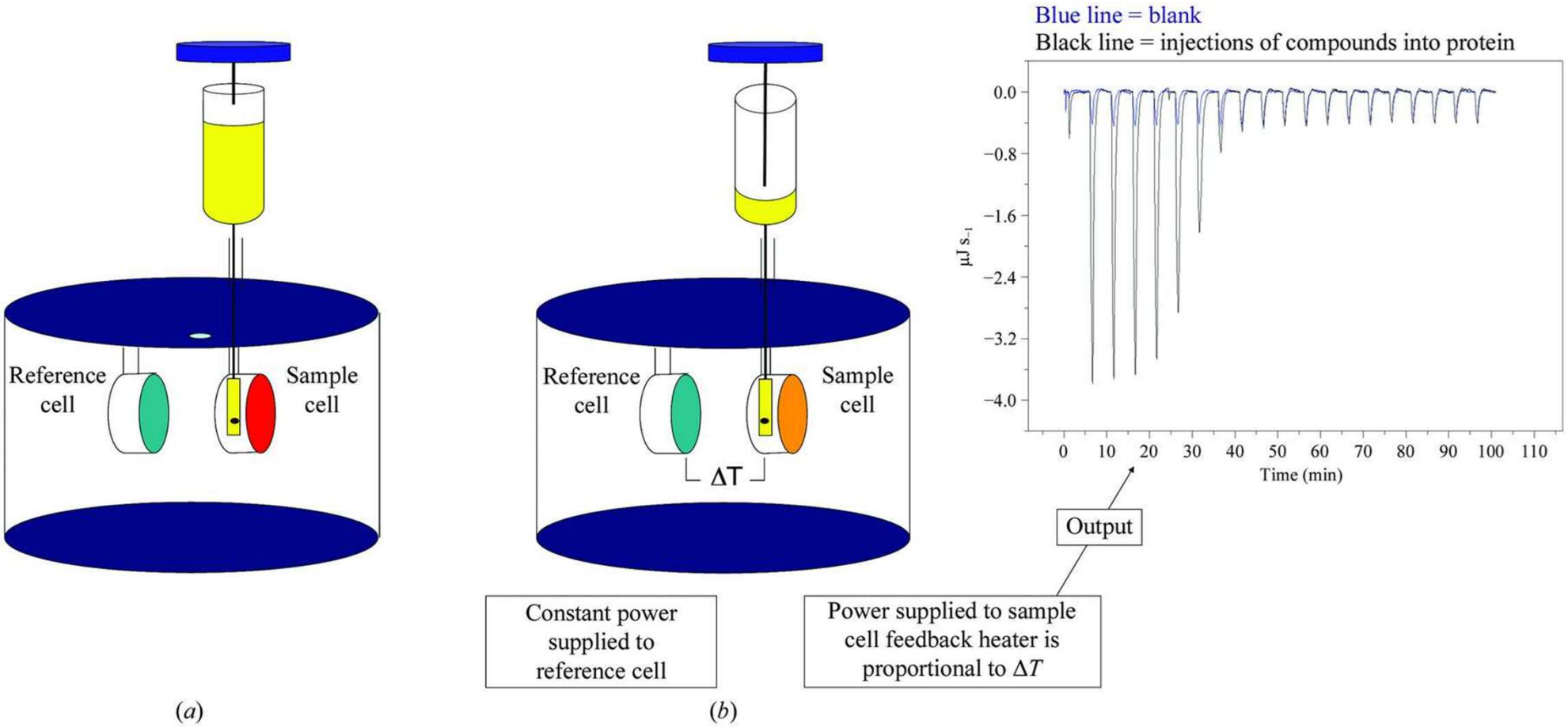




Used for:

- Protein Stability
- Conformational changes
- Ligand Binding

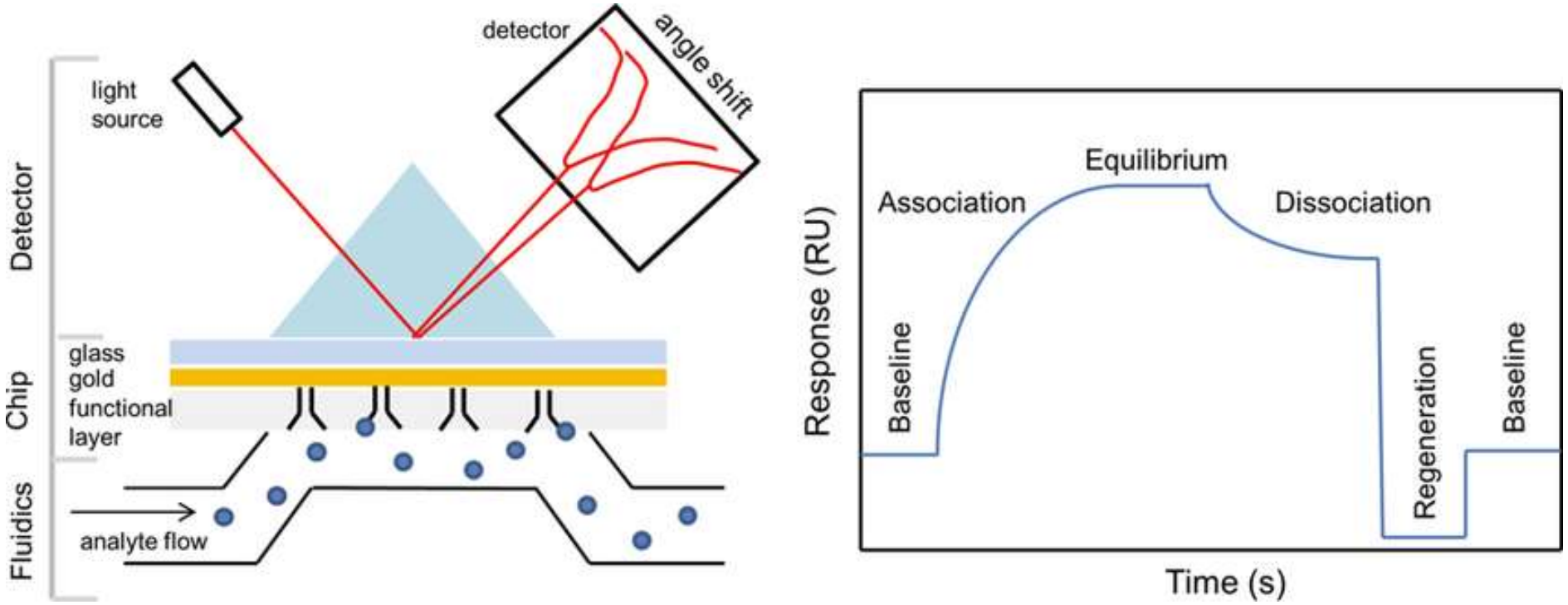
Ligand binding:
Isothermal Titration Calorimetry (ITC)



Energy supplied to the reference cell used as probe to follow binding of ligand/protein to the target protein.

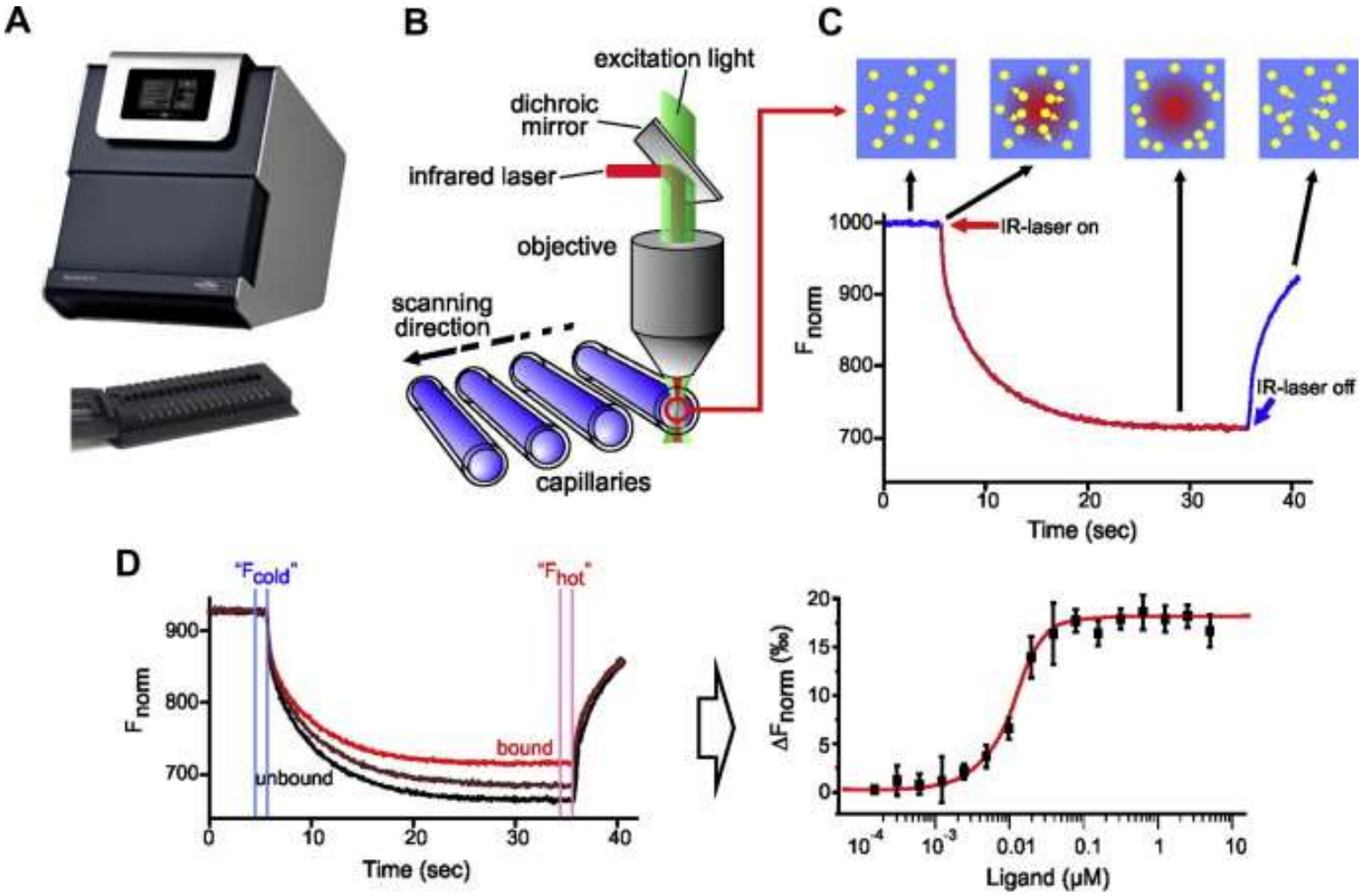
ITC experiments provide a thermodynamic picture of binding.

Ligand binding: Surface Plasmon Resonance



The SPR measures changes in the refractive index at the surface of the sensor. Molecule binding at the surface changes the refractive index proportionally to the mass bound.

Ligand binding: Microscale Thermoforesis

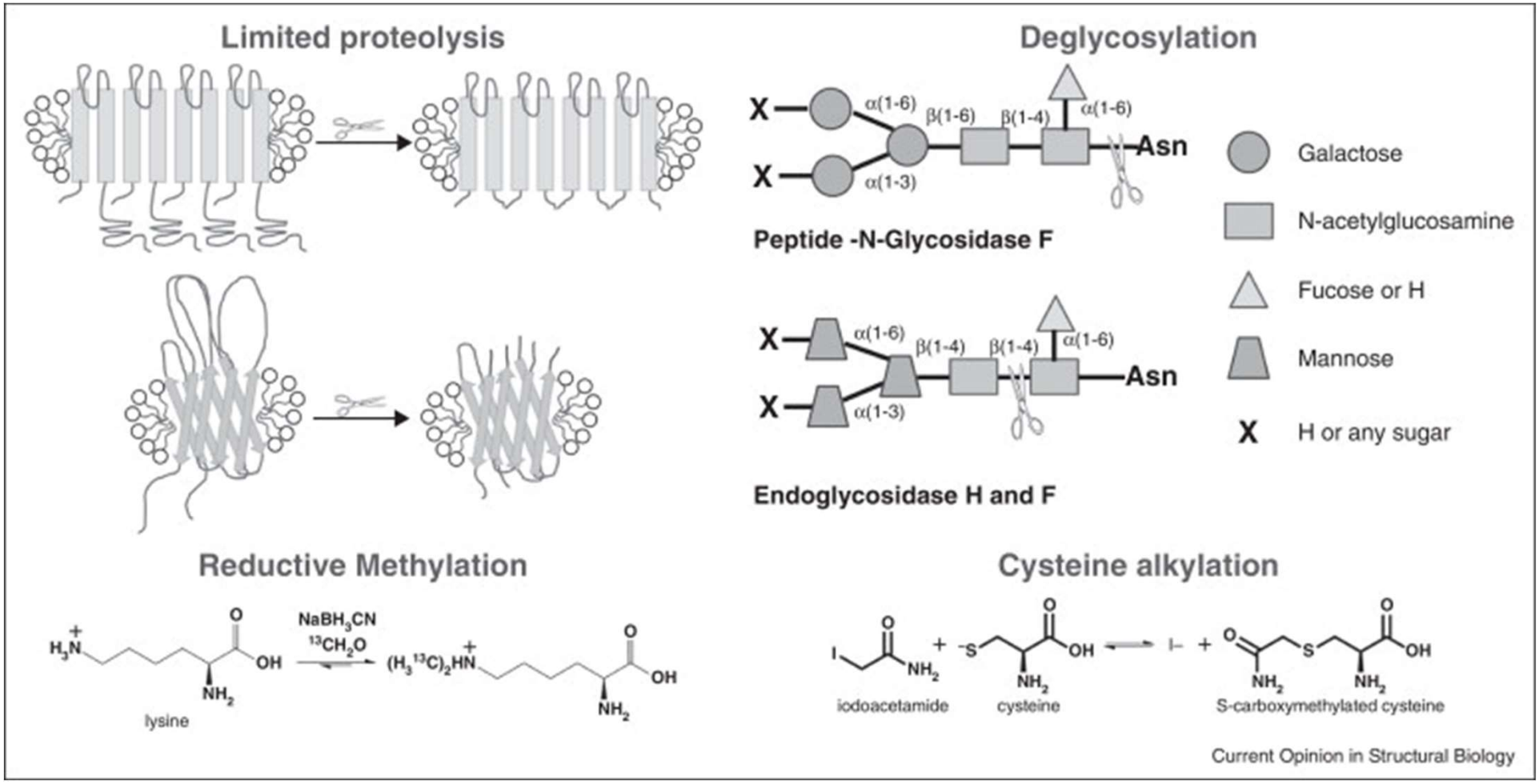


During the experiment, labeled molecules move through infrared laser-induced temperature gradients depending on their size, charge, and hydration shell.

Post-expression strategies

…recovery strategies for proteins difficult to crystallize…

Modification the protein level to improve stability, solubility, crystallizability, …



In addition… **Crosslinking** with glutaraldehyde or other chemical reagent

References

- Purification strategies: Kim Y. *et al.*, "High-throughput protein purification and quality assessment for crystallization.", **Methods 2011**, *55(1)*:12-28; Konczal J. & Gray C.H., "Streamlining workflow and automation to accelerate laboratory scale protein production.", **Protein Expr Purif. 2017**, *133*:160-169.
- **GE Healthcare Handbook**, "Purifying Challenging Proteins", http://www.gelifesciences.com/file_source/GELS/Service%20and%20Support/Documents%20and%20Downloads/Handbooks/pdfs/Purifying%20Challenging%20Proteins.pdf
- **GE Healthcare Handbook**, "Recombinant Protein Purification Handbook", https://www.gelifesciences.com/gehcls_images/GELS/Related%20Content/Files/1336168762999/litdoc18114275_20120505001440.pdf
- **Amersham Handbook**, "Protein Purification", download from Gaudet Lab website, Harvard University, http://labs.mcb.harvard.edu/Gaudet/Resources_Files/GEHealthcare_chromatography/Don%27t%20move/18113229AB.pdf
- Protein stability: Deller M.C., Kong L., and Rupp B., "Protein stability: a crystallographer's perspective", **Acta Crystallogr F Struct Biol Commun. 2016**, *72(Pt 2)*: 72–95.
- Sahin E., Roberts C.J. (2012) Size-Exclusion Chromatography with Multi-angle Light Scattering for Elucidating Protein Aggregation Mechanisms. In: Voynov V., Caravella J. (eds) Therapeutic Proteins. Methods in Molecular Biology (Methods and Protocols), vol 899. Humana Press, Totowa, NJ. https://doi.org/10.1007/978-1-61779-921-1_25
- Visvaldas Kairys et al. (2019) Binding affinity in drug design: experimental and computational techniques, Expert Opinion on Drug Discovery, 14:8, 755-768, DOI: 10.1080/17460441.2019.1623202