Biophysical techniques for HTS

InnoCore IO2.4 HTS for Drug Discovery

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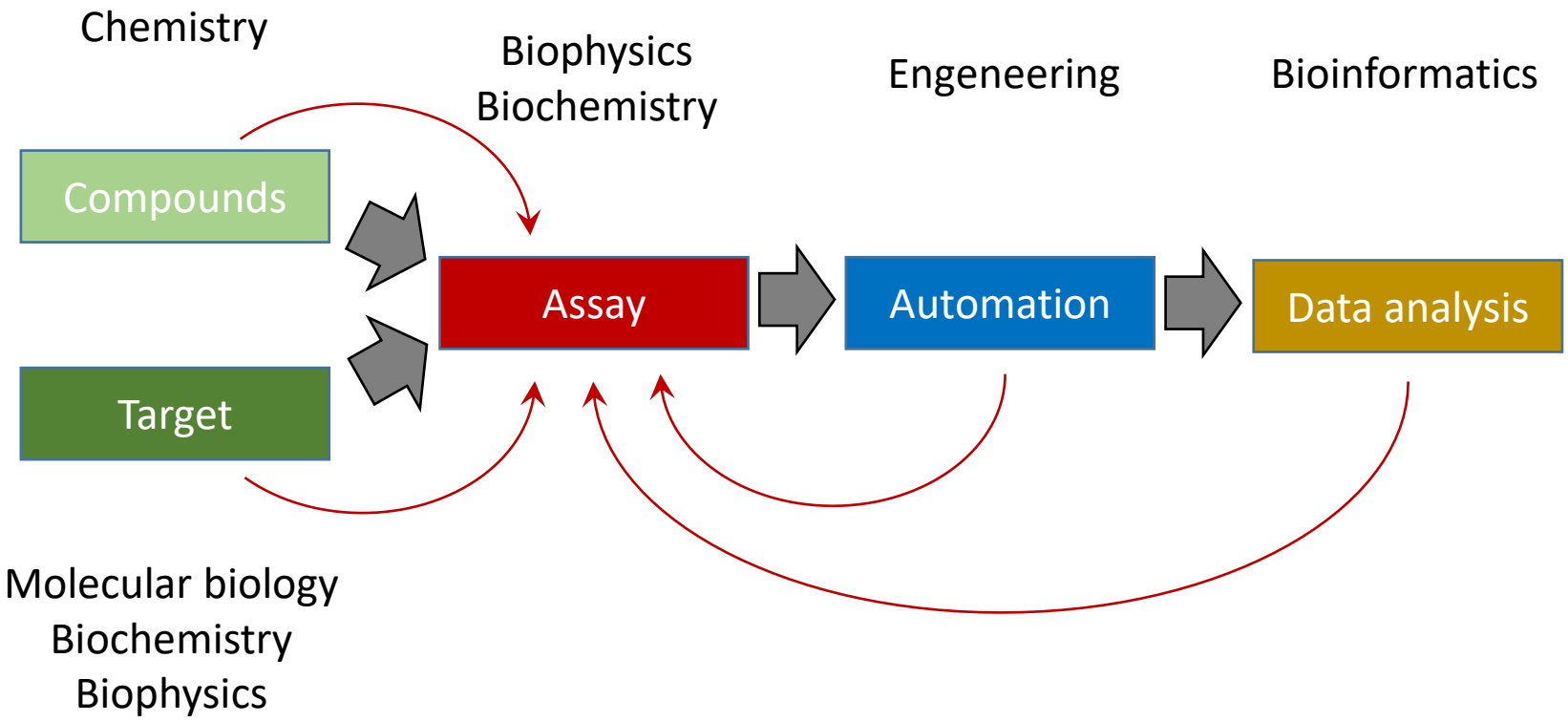
Outline

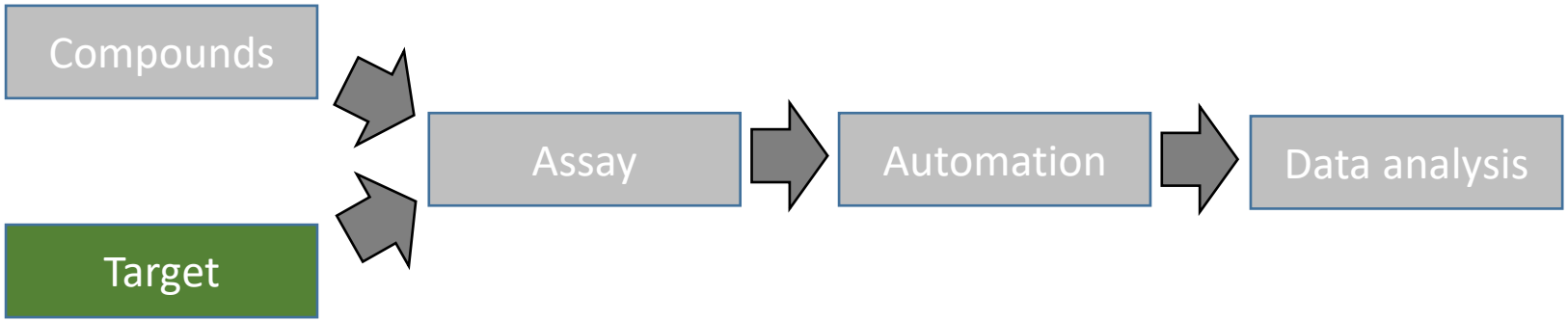
- Characterization of target molecule
- Basics of target-compound interactions
- Overview of biophysical techniques

HTS scheme

If you want to develop HTS process, you need to know as much as possible about given parts.

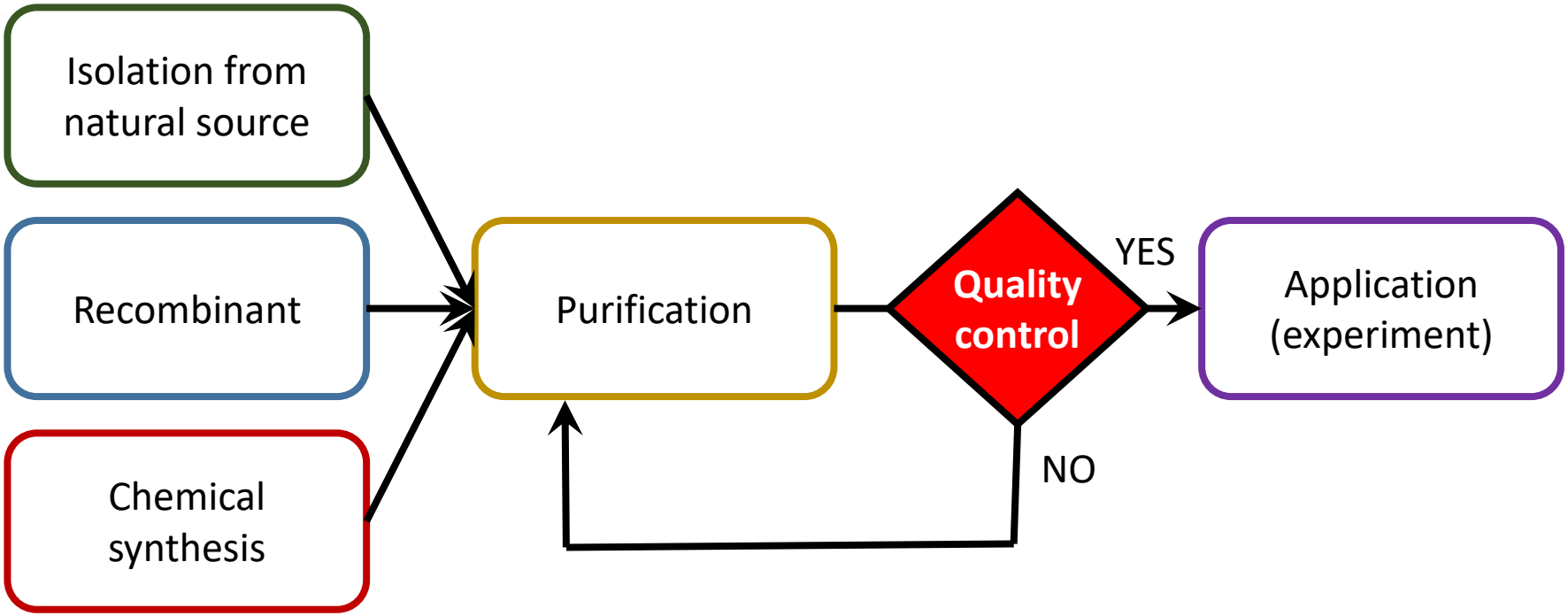


Target (biomacromolecule)



Target molecule

Typically **protein**, eventually **nucleic acid**



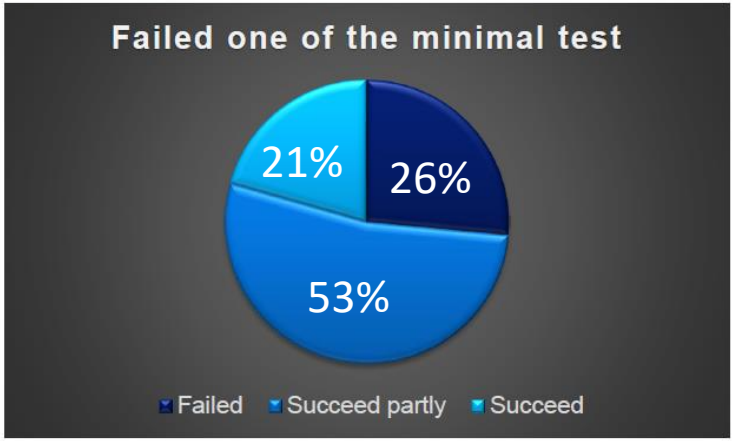
Minimal requirements for macromolecule

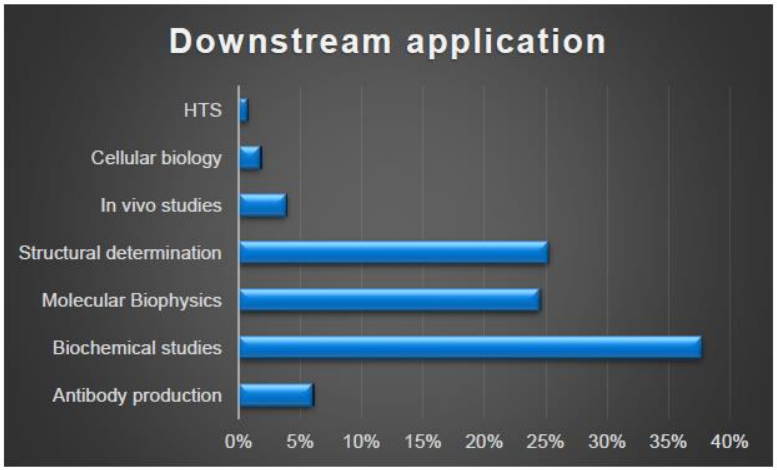
- **Identity**
  - Sequence, length (truncation), modifications, cofactors
- **Purity**
  - Contaminants – proteins, NA, polysaccharides, small molecules
- **Concentration**
  - Molar ($mol.l^{-1}$ = M), mass ($mg.ml^{-1}$), concentration of active form!
- **Homogeneity**
  - Oligomeric state, aggregation
- **Stability – folding**
  - Presence of 2D structure, stability in time, heating/ionic strength

Poor sample = Poor data

Results of the combined ARBRE / P4EU survey:

- 186 samples from 47 laboratories tested for quality
- 30% failed at least one test
- All used for some downstream application





Adapted from Arthur Sedivy, VBCF, Vienna

Experimental techniques to characterize the macromolecule

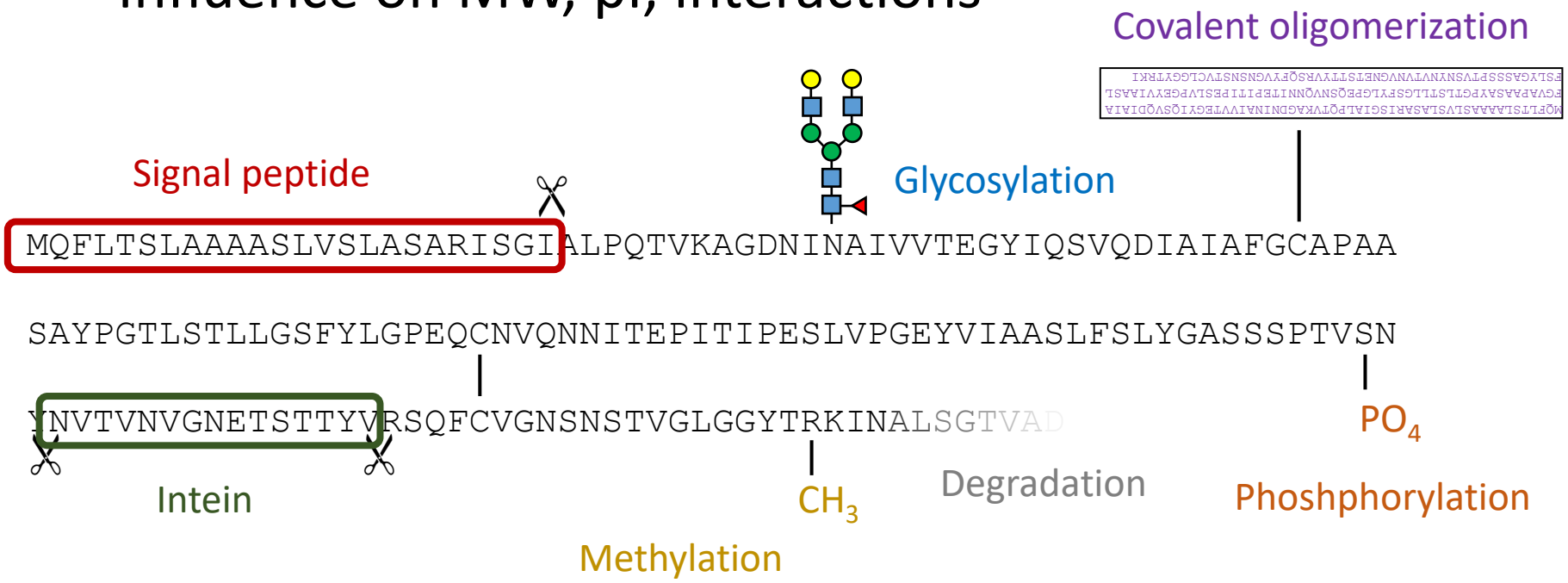
- **Identity**
  - Mass spectrometry (MS), (Immunoblotting)
- **Purity**
  - SDS-PAGE, MS, UV-VIS spectrometry
- **Concentration**
  - UV-VIS spectrometry (Trp absorbance, Bradford, Folin, peptide bond)
- **Homogeneity**
  - DLS, GPC (SEC), SEC-MALS, AUC
- **Stability – folding**
  - TSA (DSF), nanoDSF, DSC, CD, IR

Identity

Be sure what you work with

Sample identity

- Exact composition of sample (sequence, modifications, cleavage)
- Influence on MW, pI, interactions



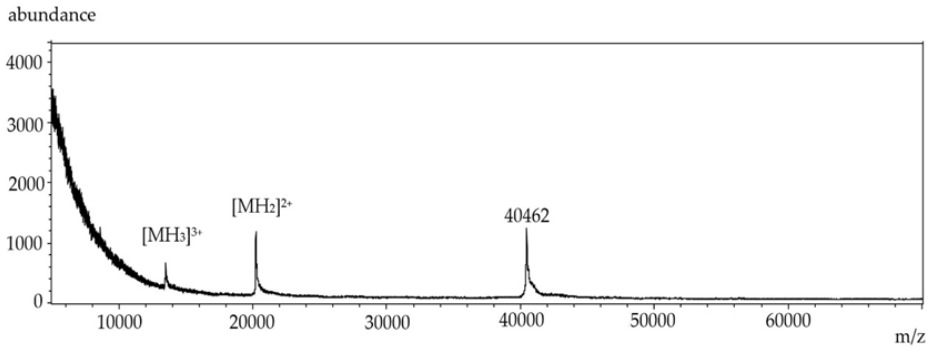
Sample identity – MS

- Detecting of exact mass of particles
- Various applications based on set-up

Identification

```
  1 MKKESINTSG PDNTKSSISD EIEISNEISW TALSGVISAA NNADGRLEVF
 51 GVGTNNAVWH NWQTVPNTGS SWSGWHSLNE GATSKPAVHI NSDGRLEVFV
101 RGTDNALWHN WQTVPGAGWS GWQSLGGQIT SNPVVYINSD GRLEVFARGA
151 DNALWHIWQT APHAGPWSNW QSLNGVLTSD PTVYVNASGR PEVFARSNDY
201 SLWYIKQTAS HTYPWTNWQS LSGVITSNPV VISNSDGRLE VFARGSDNAL
251 WHIWQVAPNA GWTNWRSLSG IITSDPAVHI NADGRLEVFA RGPDNALWHI
301 WQTATSDAWS EWTSLSGVIT SAPTVAKNSD GWLEVFARGA NNALCHIQQT
351 TSSWSTWTSL GGNLIDASAI K
```

Intact mass analysis



Purity

What accompanies your target

Sample purity – methods

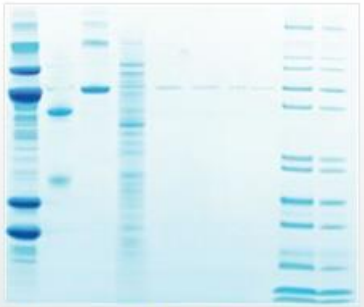
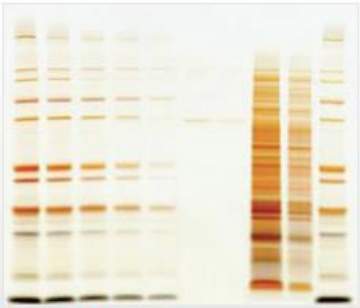
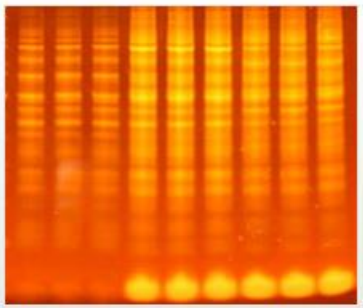
- SDS-PAGE
- UV-VIS spectroscopy
- Mass spectrometry
- SEC (SEC-MALS)
- FFF (FFF-MALS)

Small molecules:
- Co-factors
- Ligands
- Salts
- Lipids
- Saccharides

Macromolecules:
- Proteins
- Protein isoforms
- Nucleic acids
- Polysaccharides
- Binding partners

SDS-PAGE

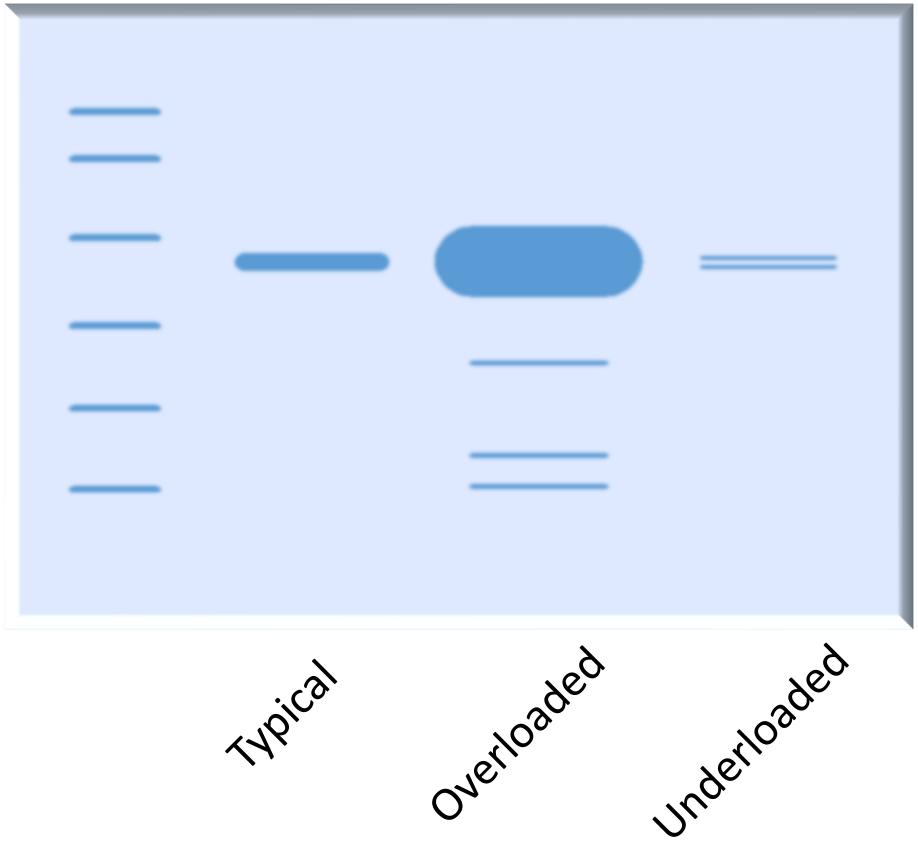
- Polyacrylamide gel (8 – 20 %)
- SDS – uniform (?) protein charge (composition dependent)
- Reducing agent (optional) – βME
- Staining – CBB, Silver, Fluorescent, Radiological

	 Coomassie staining	 Silver staining	 Fluorescent protein staining
Sensitivity	5-25 ng	0.25-0.5 ng	0.25-0.5 ng

www.thermofisher.com

SDS-PAGE

- Use overloaded as well as underloaded sample

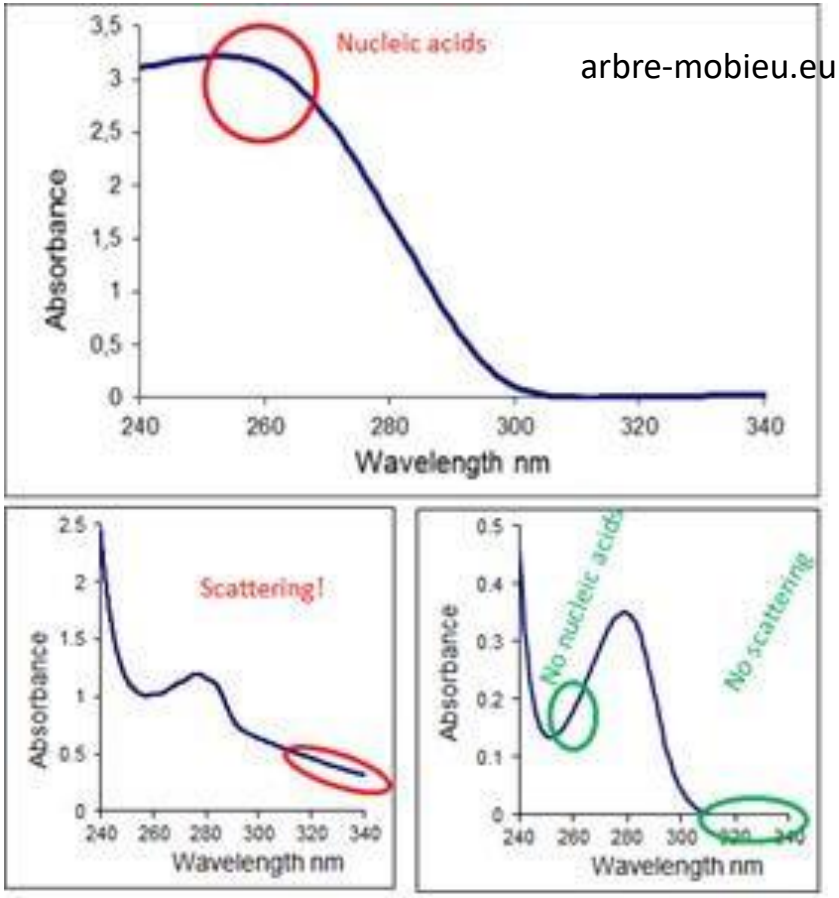


UV-VIS spectroscopy

- 240 – 340 nm
  (200 – 340 nm)

- Determination of protein/NA concentration

- Detection of:
  - Nucleic acid contamination
  - Aggregation
  - UV-absorbing contaminants



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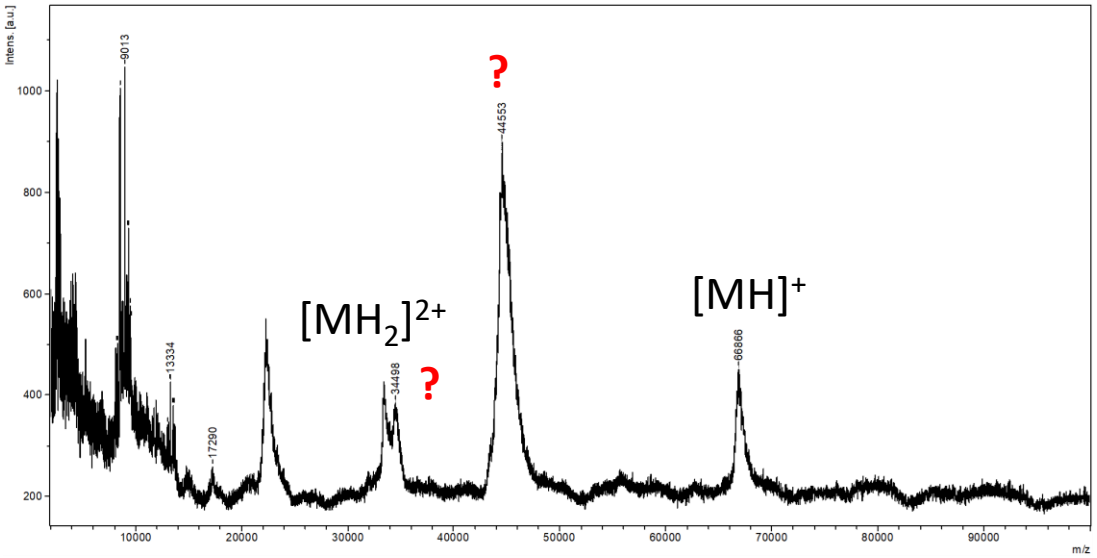
Mass spectrometry

- **Intact mass analysis –**
  protein and non-protein contaminants



Homogeneity

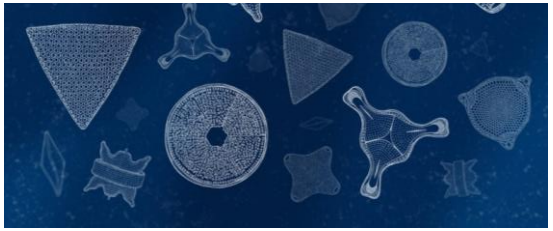
Are all target molecules identical

Sample homogeneity

- **Macroscopic** – precipitation – visual detection
- **Microscopic** – oligomeric states, folding states, microheterogeneity – biophysical methods

Macroscopic

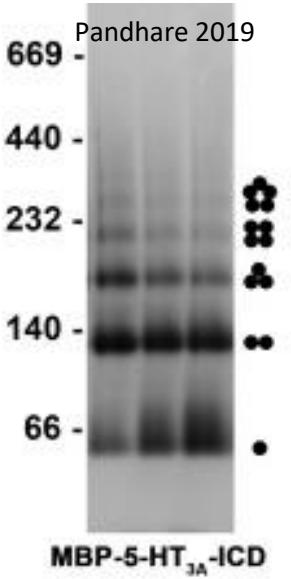
Heterogeneity



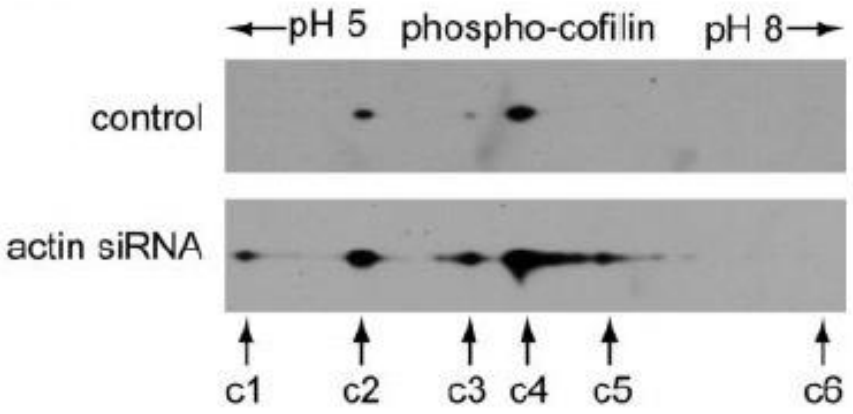
Microscopic

Native electrophoresis

- Possibility to observe various **oligomers** (relatively imprecise and unreliable) and **isoforms** (2D PAGE preferred)
- Not efficient for **aggregation** detection



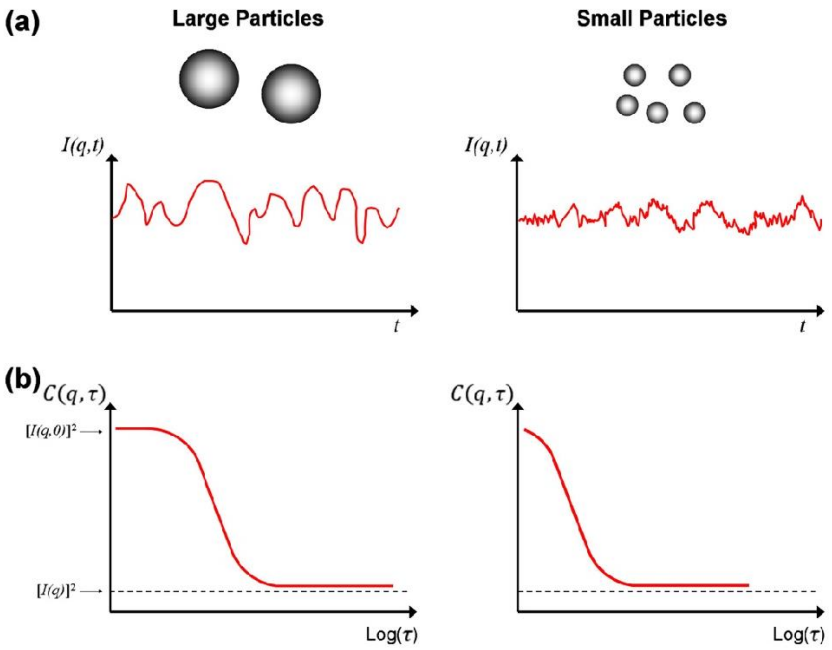
Pandhare 2019

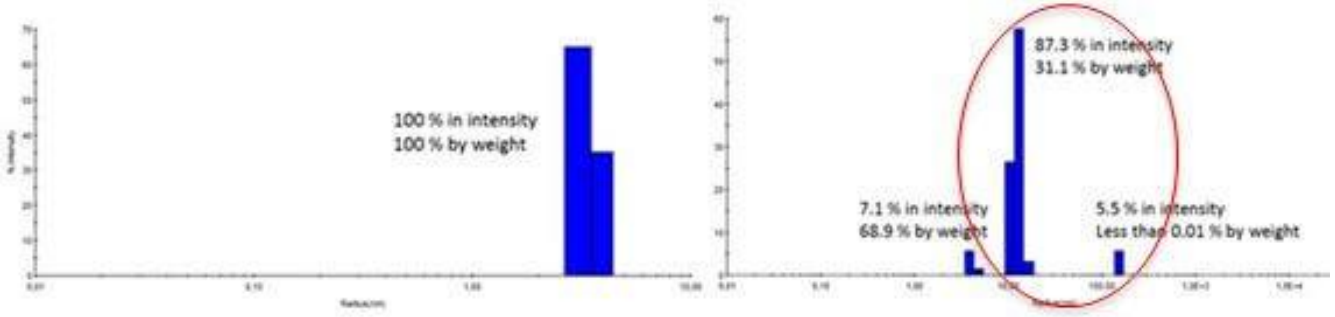


Light scattering

- **Dynamic light scattering**
  – size of particles

- **Static light scattering**
  – mass of particles





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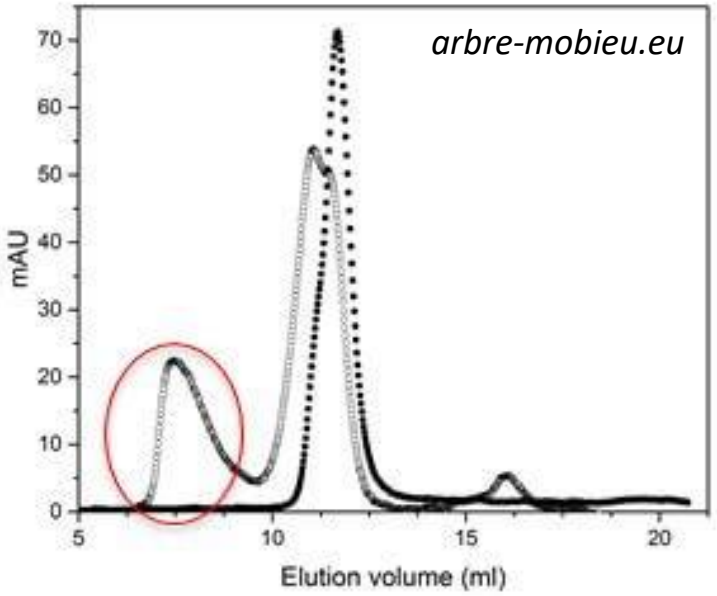
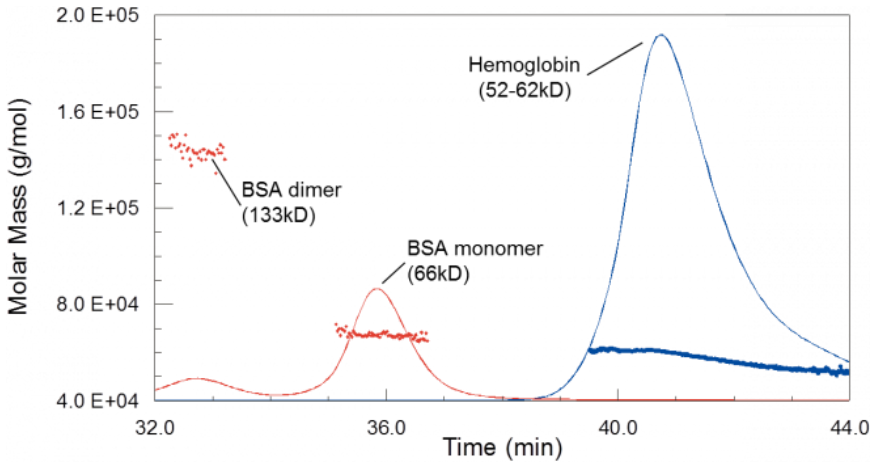
DLS is highly sensitive to aggregation

Size exclusion chromatography

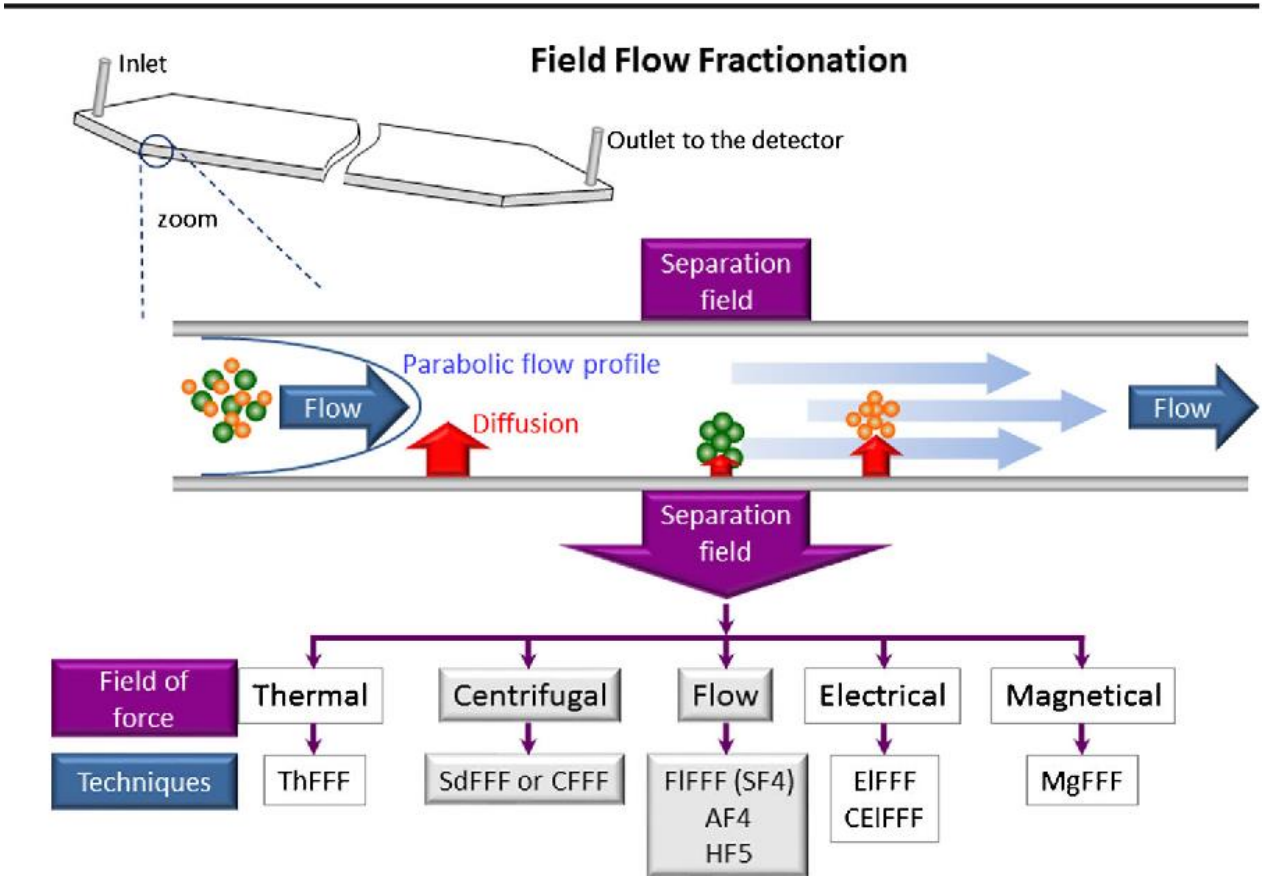
- Separation of particles based on “size”
- Interaction with matrix possible (!)
- Frequently coupled to multiple detectors
  (UV, MALS, RI, viscosity)





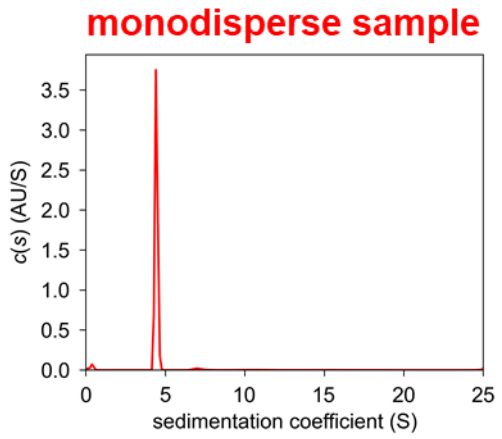
Field flow fractionation

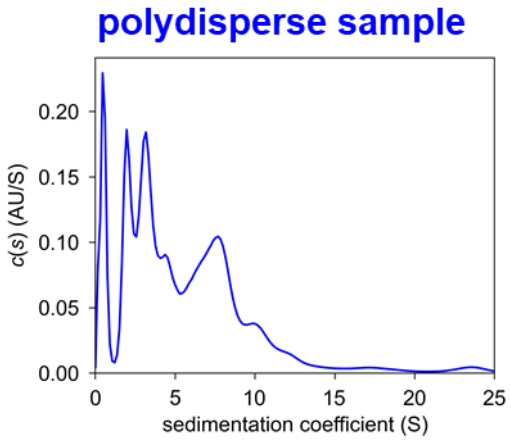
- Separation of particles in solution by **external** force **field**



Analytical ultracentrifuge

- Separation of particles in **centrifugal field** by hydrodynamic properties
- Two modes:
  - Sedimentation equilibrium – mass determination
  - **Sedimentation velocity** – size distribution
- Sensitive to oligomers and aggregates





Stability – Folding

Is the molecule folded and "happy"

Sample stability

Tendency to retain state (structure) with change of conditions

- **Temperature** stability
- **Chemical** stability
  - pH
  - ionic strength
  - oxidizing agents
  - additives
- Long-term stability – **storage**

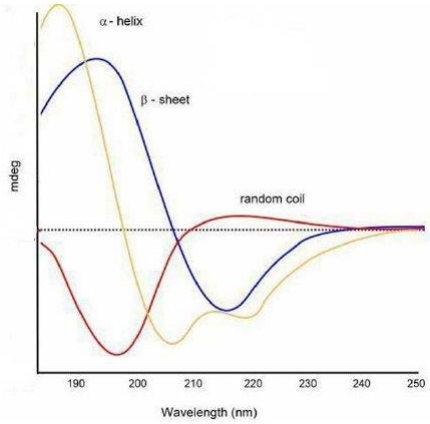
Buffer choice is important !

Buffer optimization

- Batch-to-batch **quality check**

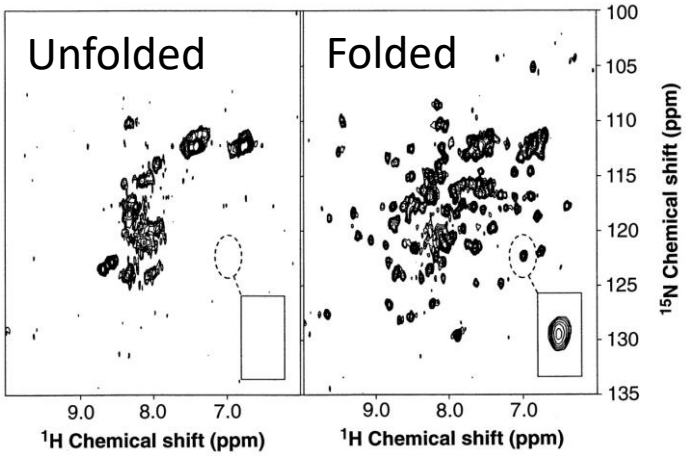
Folding – direct evidence of 2D structure

- **Circular dichroism** (CD)
  - Difference in absorption of left and right circularly polarized light by chiral compounds
  - Specific shape of spectra for 2D structural elements



Dodero 2011

- **Nuclear magnetic resonance** (NMR)
  - Behavior of atom nuclei in magnetic field
  - Presence of defined structure results in distinguished peaks in spectrum



Balbach 1996

Temperature

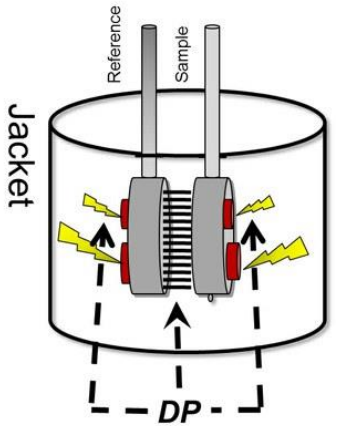
- Affects stability and interaction parameters

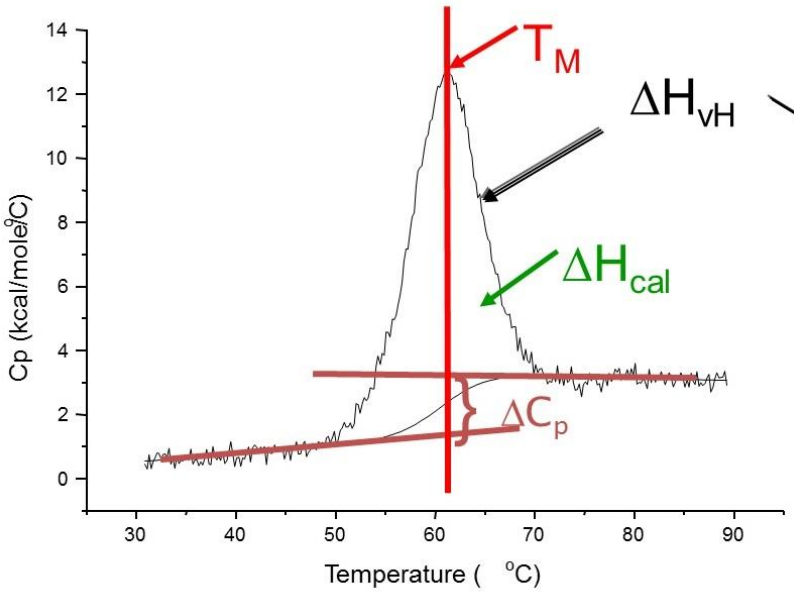
$$K_A = e^{\frac{RT}{\Delta G_0}}$$

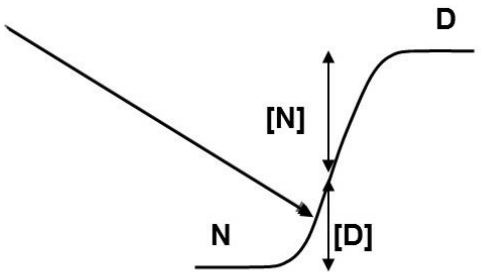
- Typical temperatures:
  –80 °C, –20 °C, 4 °C, 20 °C, 25 °C, 37 °C
- *Room temperature* (RT) – vaguely defined
  mostly 20 – 25 °C, but varies from 15 – 30 °C
  usually means that temperature was not set (!)

Differential scanning calorimetry (DSC)

- Measures **changes in heat** over a range of temperatures
- Direct assignment of protein T_m, ΔH_{cal}, ΔH_{vH}, ΔC_p – complex information

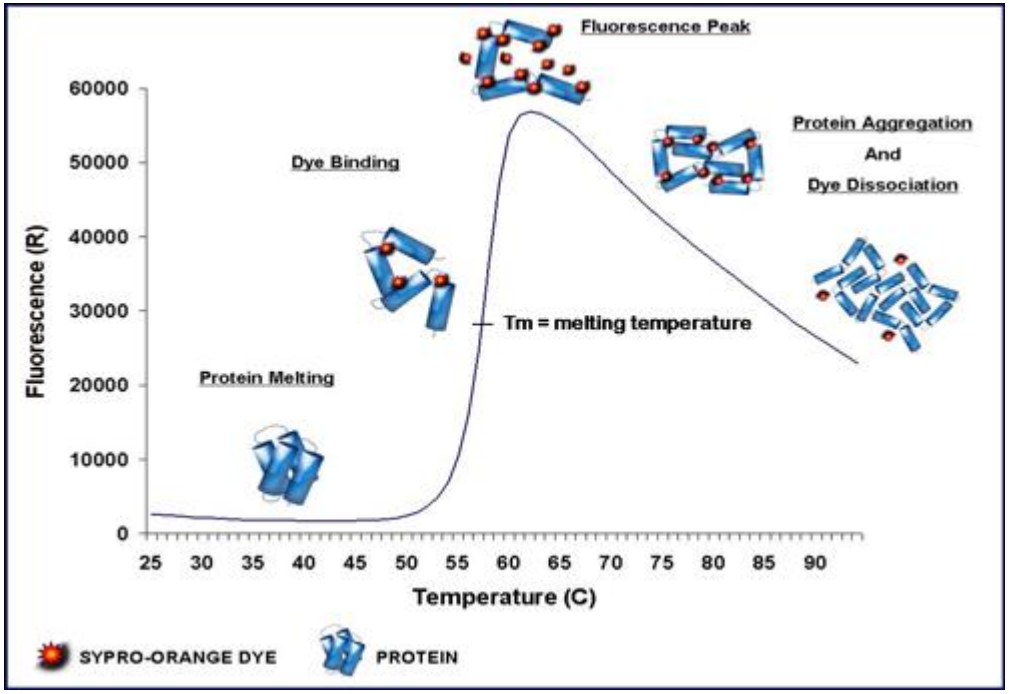






Thermal shift assay (TSA)

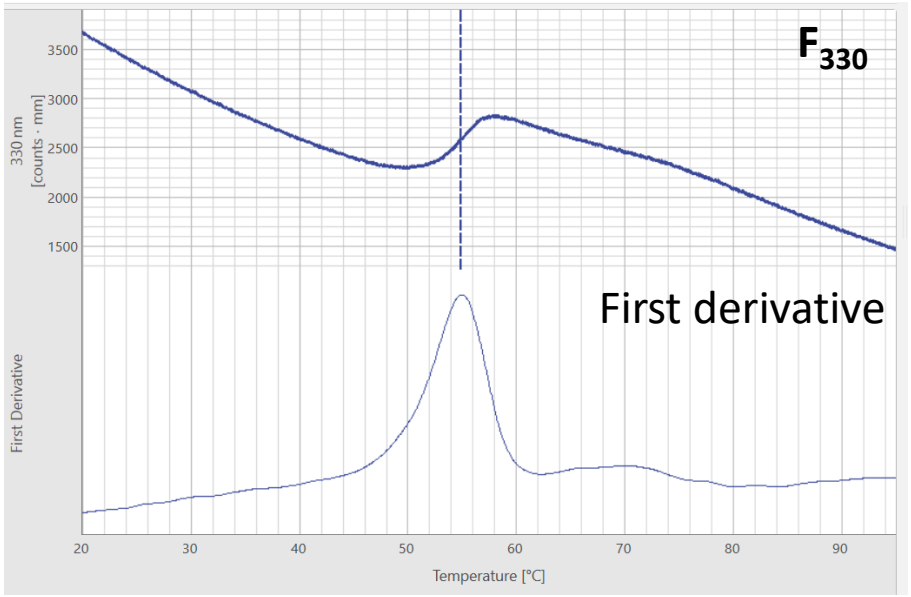
- **Fluorescent dye** binds dominantly to unfolded state.
- Fluorescence increase upon binding
- Measured in RT-PCR machine

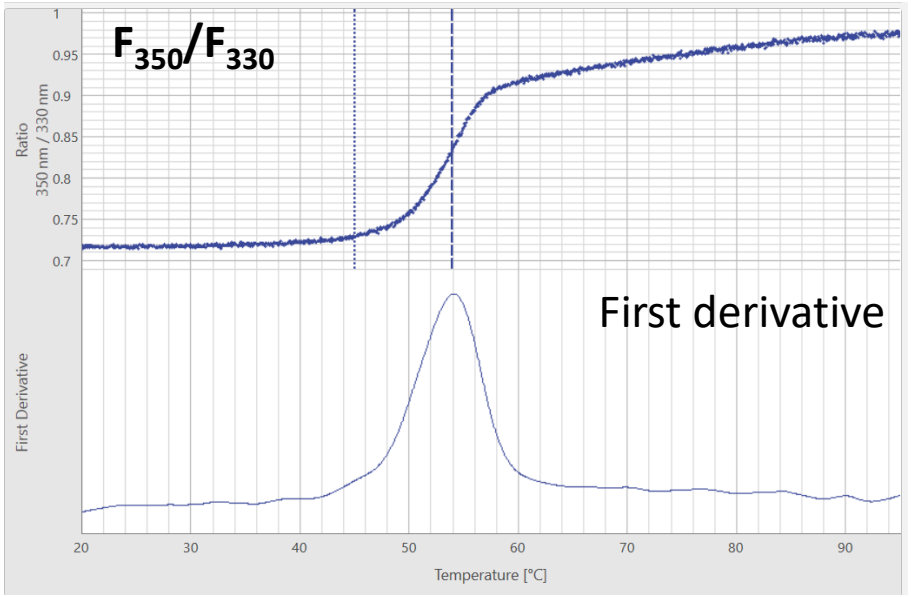


wikipedia.org

Differential scanning fluorimetry (nanoDSF)

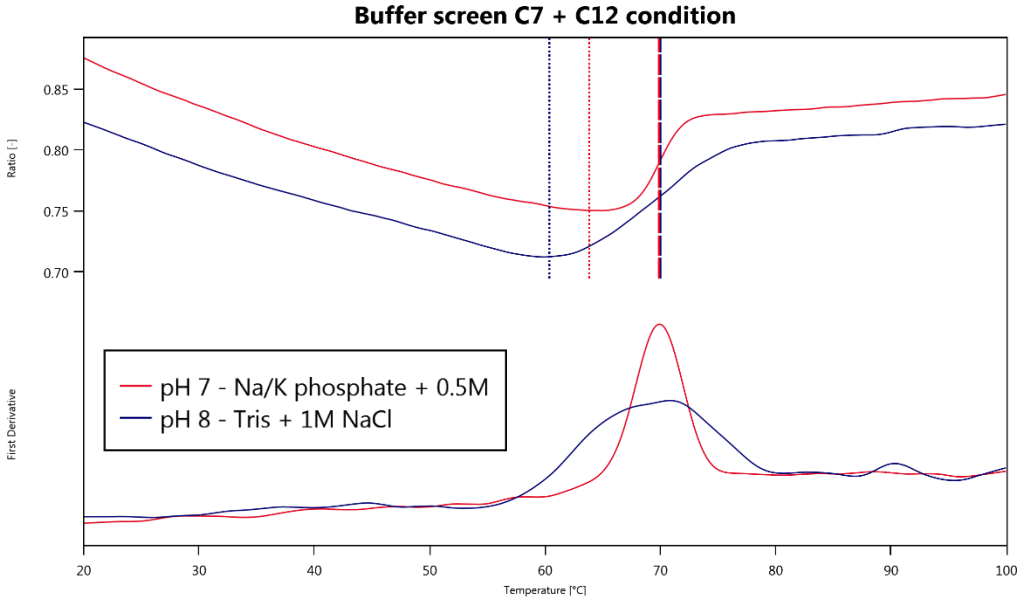
- Observation of changes of intrinsic **Trp fluorescence** (absolute or wavelength shift).
- Caused by change of microenvironment around Trp residues upon unfolding.
- No dye added – minimal interference





Buffer optimization – nano DSF test

	1	2	3	4	5	6	7	8	9	10	11	12
A	59.2°C	-	43.6°C	37.7°C	55.0°C	61.3°C	59.8°C	62.1°C	55.5°C	59.0°C	33.4°C	33.2°C
B	36.5°C	42.1°C	48.3°C	52.2°C	55.0°C	58.5°C	66.2°C	66.4°C	58.7°C	59.4°C	63.1°C	63.3°C
C	57.2°C	59.2°C	62.7°C	62.1°C	67.0°C	68.1°C	69.9°C	66.5°C	60.2°C	61.8°C	66.5°C	70.0°C
D	60.6°C	58.5°C	69.4°C	63.4°C	46.2°C	55.2°C	58.2°C	54.5°C	59.2°C	59.5°C	-	59.2°C



Original buffer **59.2**

vs.

Best buffer **69.9**

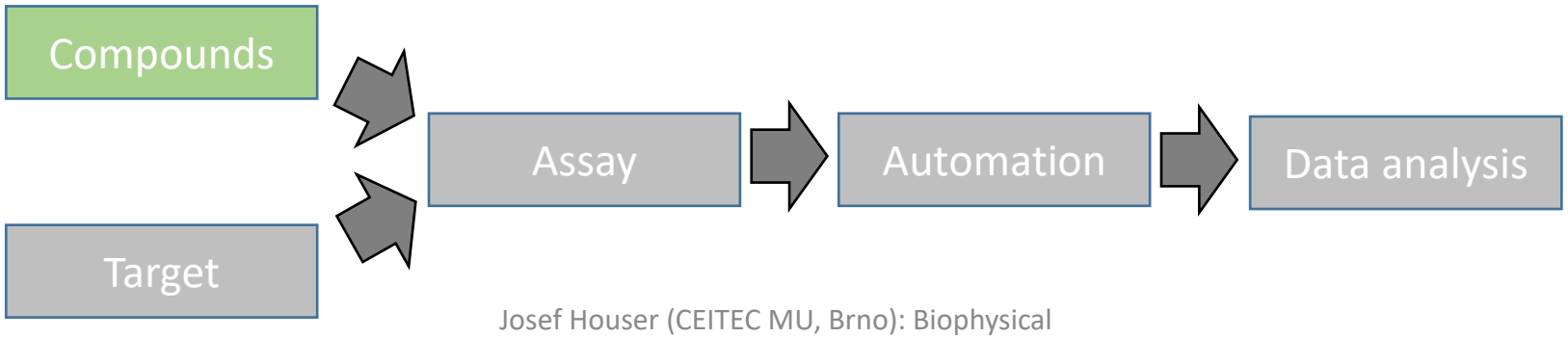
> 10°C difference !!!

Sample storage

- Depends on sample stability
- *Freezing* (phase transition) may decrease protein stability in solution
  avoid repeated freeze-thaw cycles !

- Fridge: 4 °C
- Freezer: – 20 °C, – 80 °C
  (**cryo-protectants** added – glycerol, ethylene glycol, saccharose, trehalose)
- Lyophilization = Freeze-drying: water sublimation

“Bullets”
(ligands, compound library)



Drugs and biomacromolecules

Drug can be anything that **interacts** with biological molecule

Note: If it does not, it can be still drug – Placebo effect

Molecule type:	*Drug example:*
Protein	Antibodies
Nucleic acid	siRNA
Peptide	Antimicrobial peptides
Polysaccharide	Hyaluronic acid
Small organic molecule	Paracetamol
Organo-metallic compound	Cisplatin
Inorganic molecule	Lithium carbonate
Solvent	Ethanol (antidote)

Drugs and biomacromolecules

Tested molecules have their own properties and limitations

Size: big (proteins, nucleic acids, nanoparticles)

small (inorganic and organic molecules)

Solubility: hydrophobicity (organic compounds)

pH-dependency (proteins, metallic compounds)

Temperature stability: proteins

Purity of synthesis: polysaccharides, organic molecules

Homogeneity: aggregation (proteins), clustering

Types of interaction

- **Nuclear physics**

  interaction of subatomic particles (nuclear fusion, radioactivity) 10^6 kJ/mol

- **Chemistry** (electron ionization)

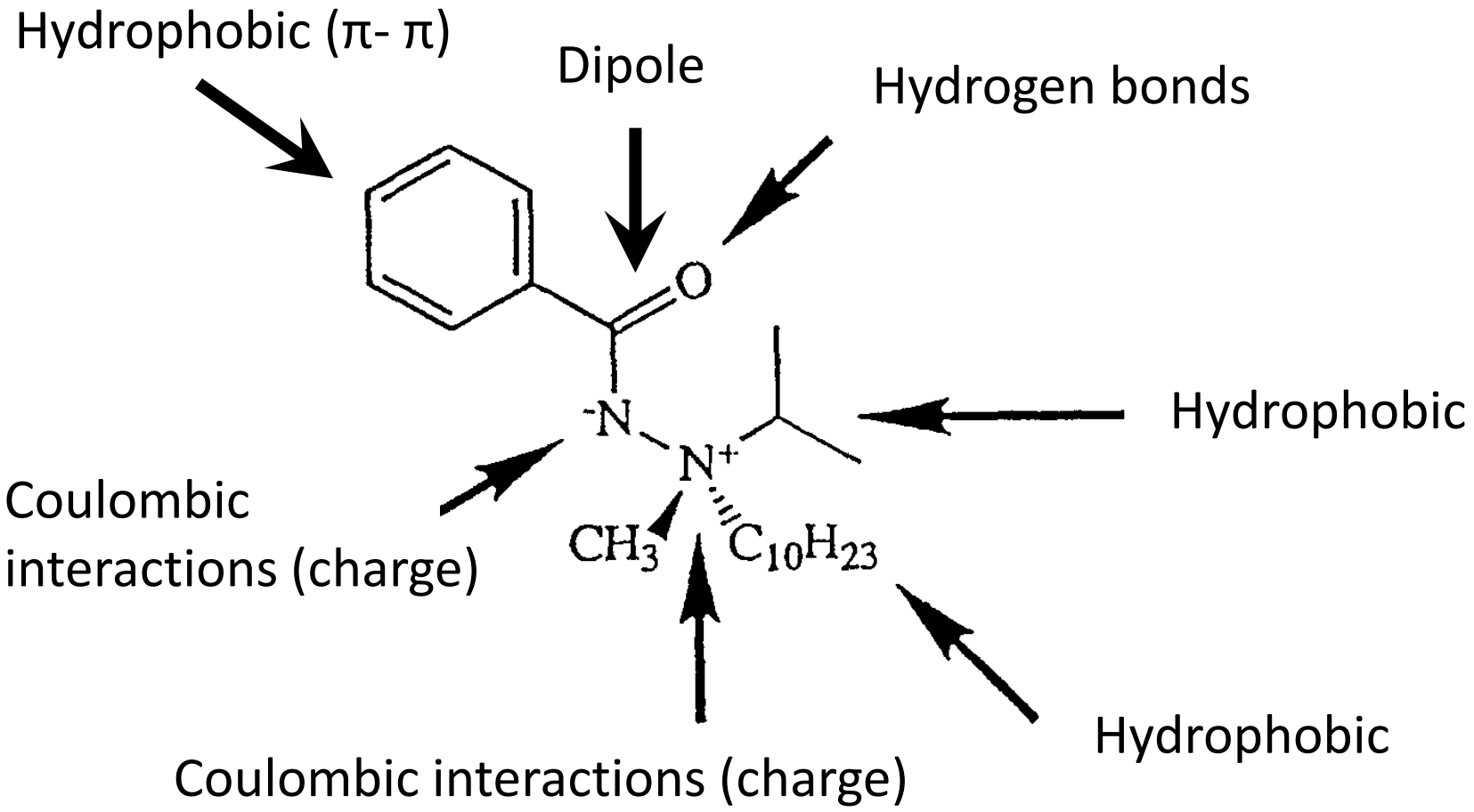
  formation of bonds 150-1000 kJ/mol

- **Biochemistry-biology**

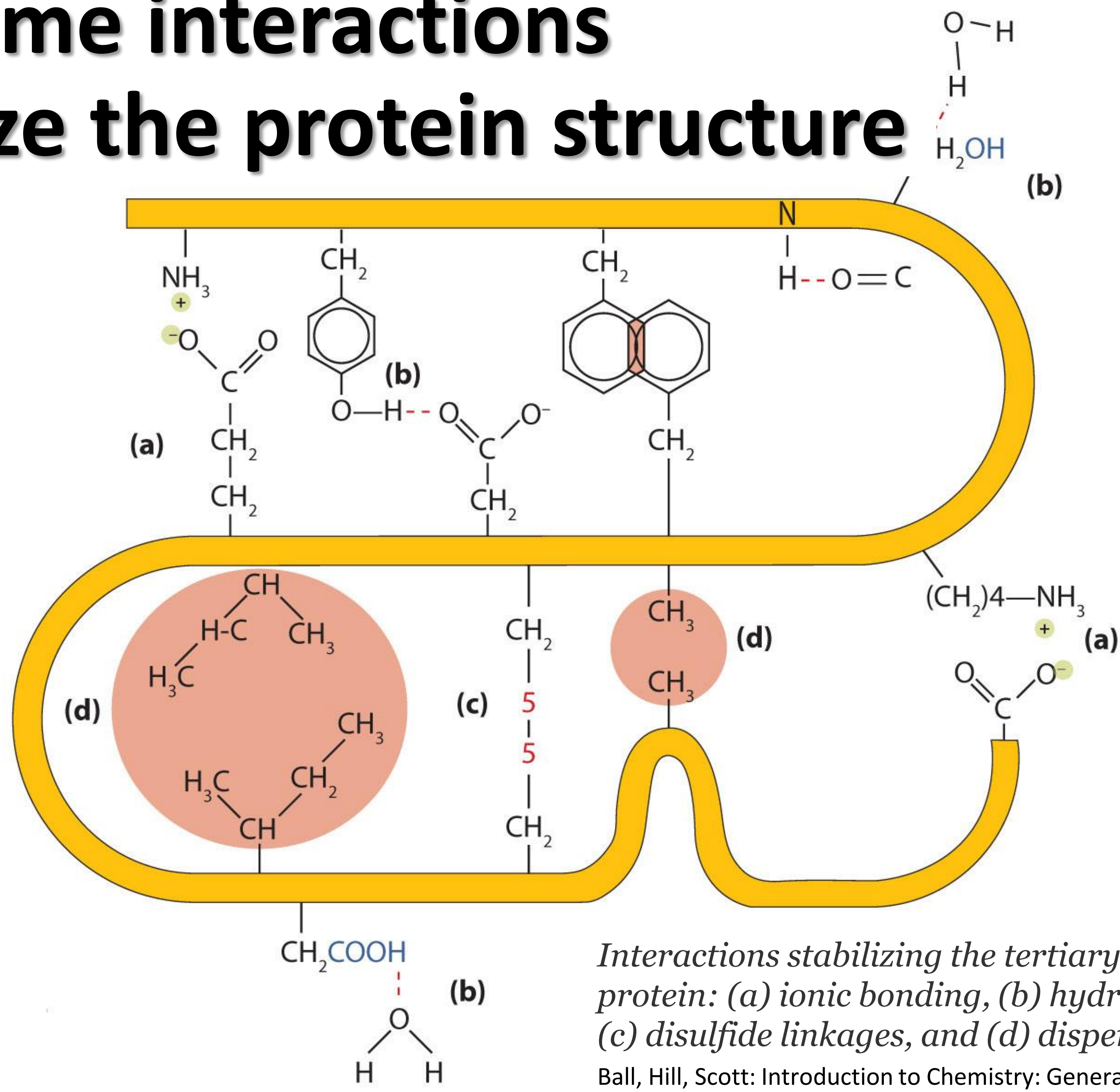
  spectrum of weak interactions (e.g. H-bond 8-30 kJ/mol)

  coulombic (salt bridge), dipoles, H-bond, hydrophobic

Mostly more than one effect is present



The same interactions stabilize the protein structure



Interactions stabilizing the tertiary structure of a protein: (a) ionic bonding, (b) hydrogen bonding, (c) disulfide linkages, and (d) dispersion forces.

Ball, Hill, Scott: Introduction to Chemistry: General, Organic, and Biological

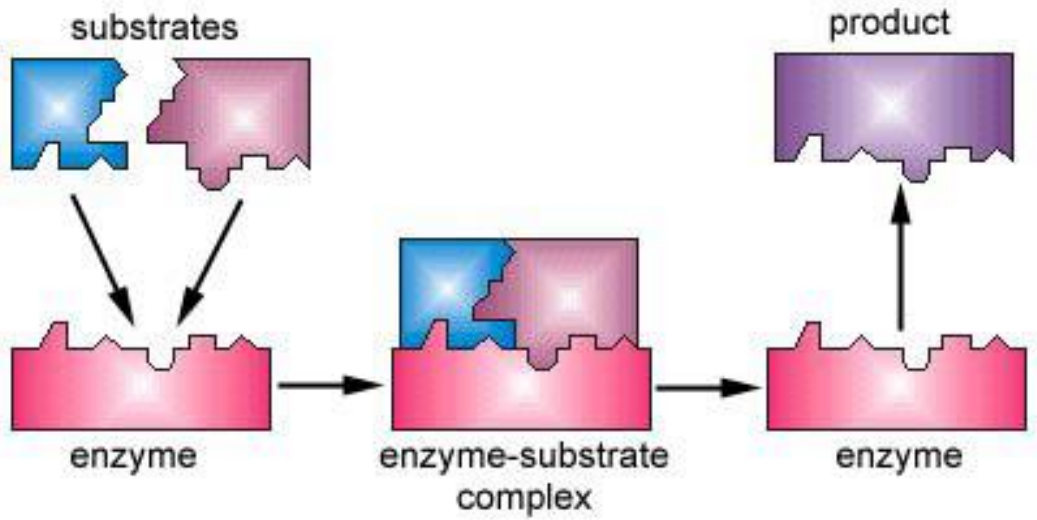
Why to study the interactions

- **Understanding of biological processes**
  - Does it bind?
  - How strong is the interaction?
  - Is the interaction influenced by temperature/additives?

- Analyzing the **nature of intermolecular interaction**
  - What type of interaction is present (hydrophobic, H-bonds, salt bridges)?

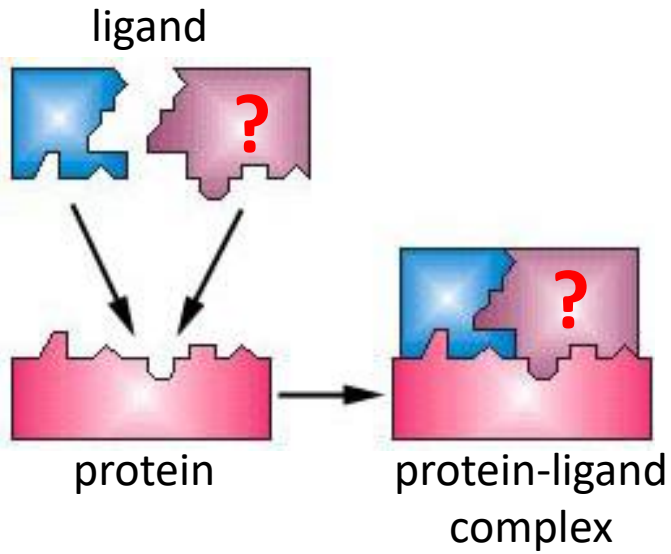
- **Application** of the knowledge in science/medicine
  - Disease pattern discovery
  - Drug development
  - Biotechnology

Interaction vs. chemical reaction



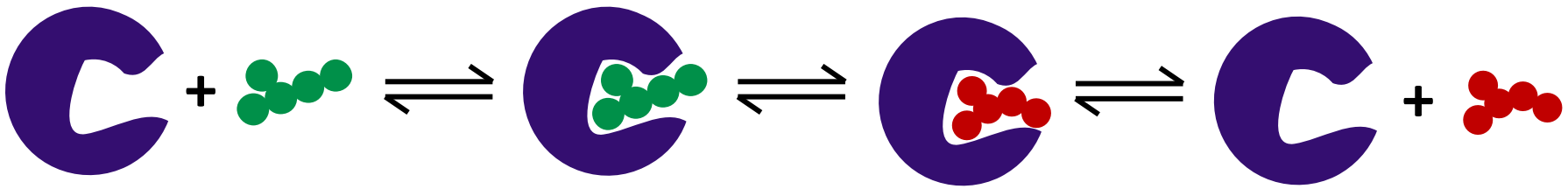
Enzyme
(Ribozyme)

Interaction vs. chemical reaction

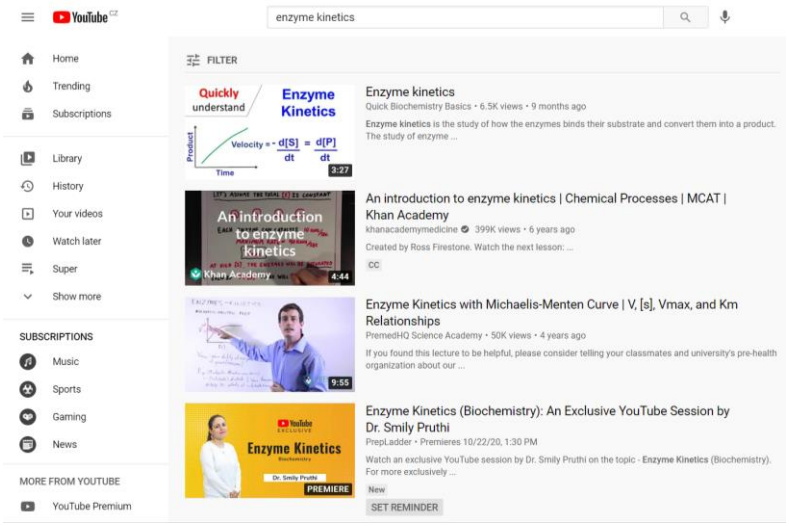


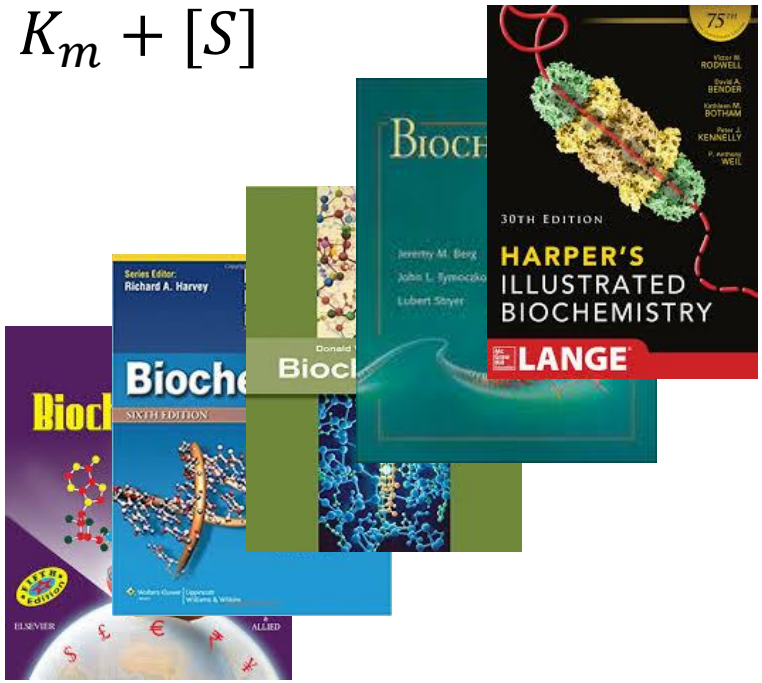
Antibody – Antigen
Receptor – Ligand
Transporter – Ligand
Lectin – Carbohydrate
Transcription factor – Nucleic acid

Enzyme-catalyzed reaction

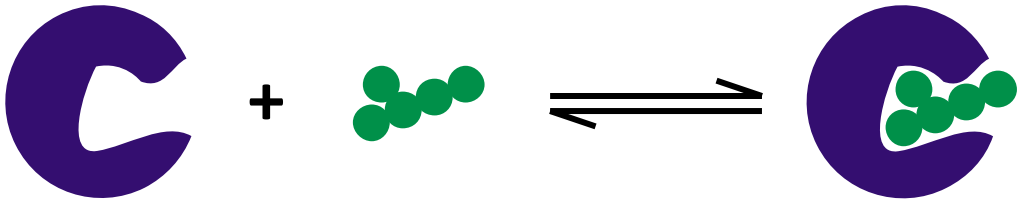


$$v = \frac{d[P]}{dt} = -\frac{d[S]}{dt} = \frac{V_{max}[S]}{K_m + [S]}$$





Receptor-ligand interaction – kinetics

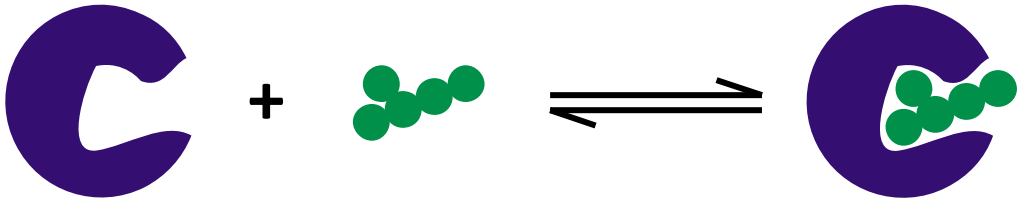


$$\frac{d[\mathrm{MX}]}{dt} = k_a[\mathrm{M}][\mathrm{X}] - k_d[\mathrm{MX}]$$

$$equilibrium: \frac{d[\mathrm{MX}]}{dt} = 0$$

$$\mathrm{K_D} = \frac{1}{\mathrm{K_A}} = \frac{k_d}{k_a} = \frac{[\mathrm{M}][\mathrm{X}]}{[\mathrm{MX}]}$$

Receptor-ligand interaction – thermodynamics



$$P + L \underset{K_D}{\overset{K_A}{\rightleftharpoons}} PL$$

$$\Delta G^\circ = -RT \ln K_A = RT \ln K_D$$

$$\Delta G^\circ = \Delta H^\circ - T\Delta S^\circ$$

$\Delta G < 0$ exergonic	$\Delta H < 0$ exothermic
$\Delta G > 0$ endergonic	$\Delta H > 0$ endothermic

Enthalpy (H)

Changes in the heat

Structure of complex

- **H-bonds**
- **Van der Waals**

Structure of solvent

- **water**

Entropy (S)

Changes in the organization

Independent rotational and translational degrees of freedom

- **Complex is more ordered than two free molecules**

Internal conformational dynamics

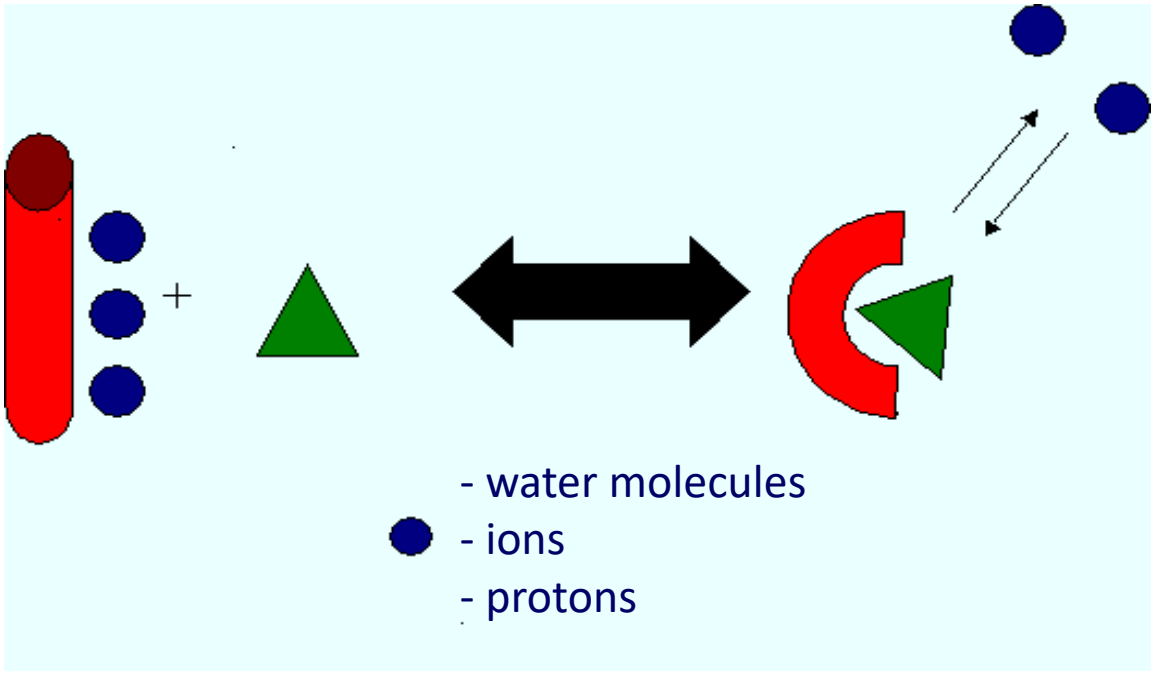
- **flexible molecules loose the entropy upon binding**

Solvent dynamics

- **water**

$$\Delta G^\circ = \Delta H^\circ - T\Delta S^\circ$$

Rational drug design – Energetic contributions involved



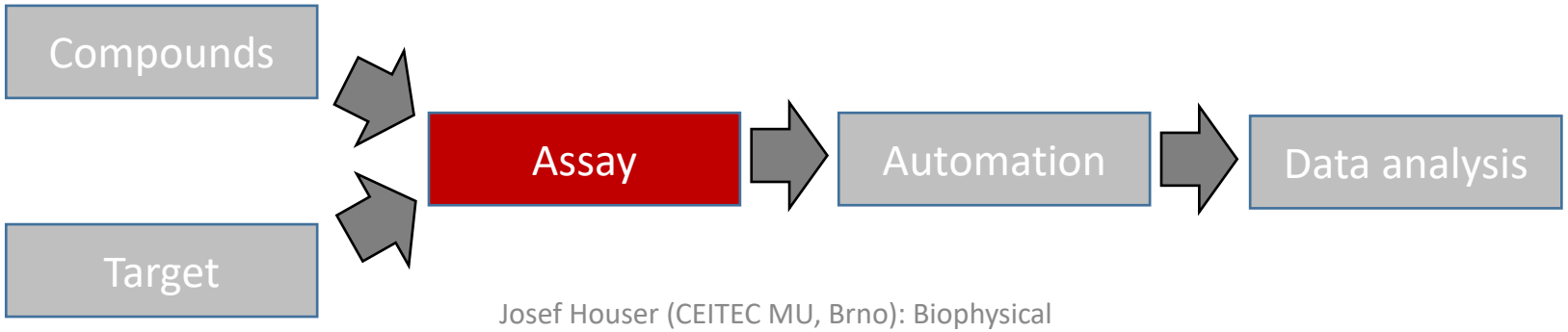
Entropy

- Hydrophobic interactions
- Water release
- Ion release
- Confromational changes

Enthalpy

- Hydrogen bonds
- Protonation

Methods (biophysical)

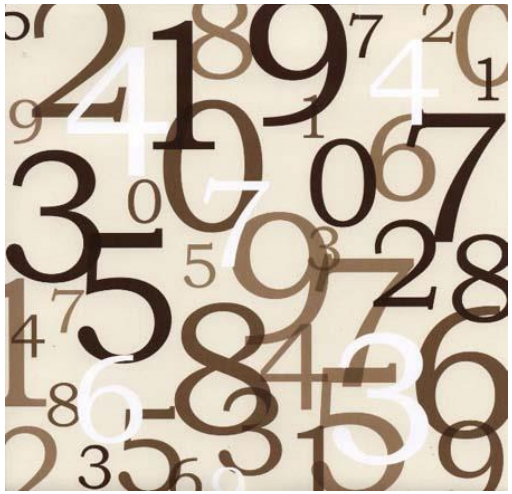


Two informational levels of methods

Qualitative

Semi-quantitative

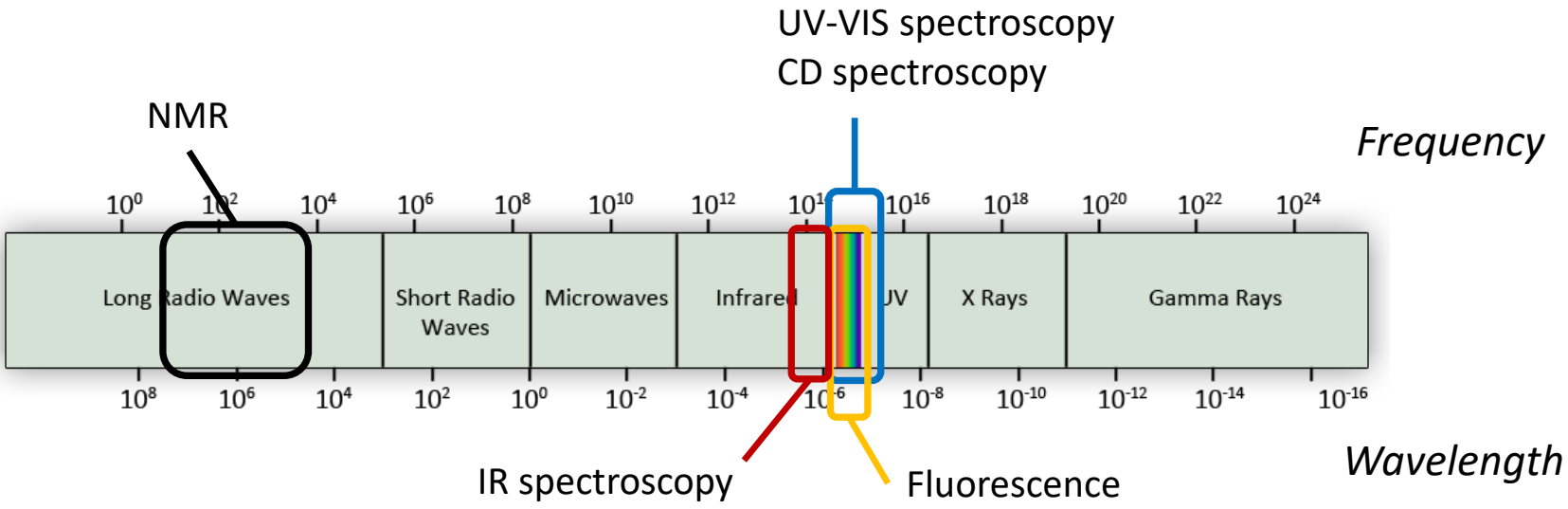
Quantitative



Experimental techniques to measure interactions

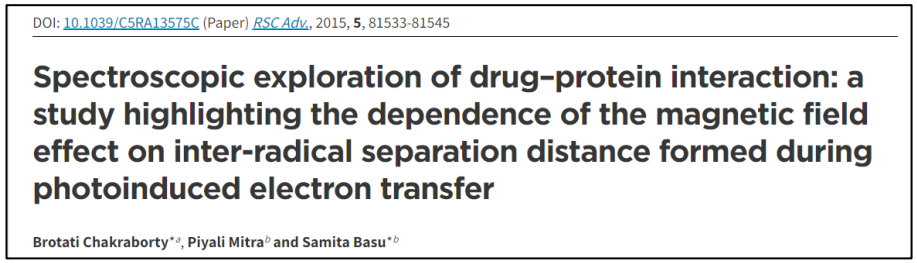
- **Optical**
  - Spectroscopy (UV, NMR, CD, IR, Raman,...)
  - Fluorescence (direct, FA, FRET, TRIC,...)
  - Biosensors (SPR, BLI,...)
- **Mobility-based**
  - Mass spectrometry, chromatography, electrophoresis, equilibrium dialysis, FFF, MST, AUC, FIDA,...
- **Calorimetrical**
  - Isothermal (ITC)
  - Scanning (DSC)
- **Stability-based**
  - DSF, DSC, CD,...
- **Structure-based**
  - X-ray, Cryo-EM
- **Electrochemical**
  - Amperometry, potentiometry, conductometry, polarography,...

Optical methods



Ultraviolet-visible spectroscopy (UV-VIS)

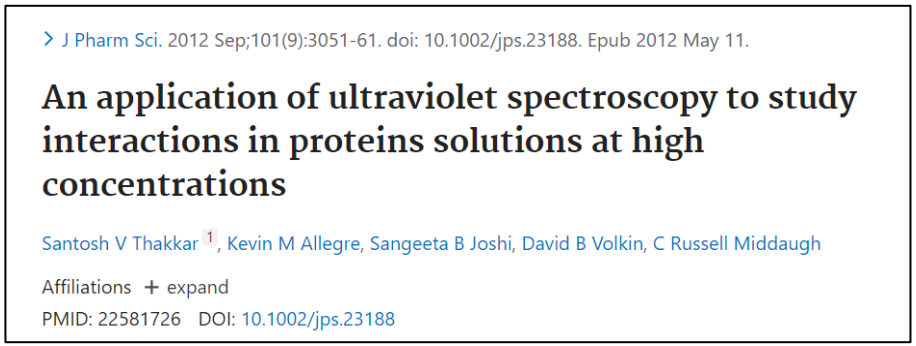
- Changes in light absorption upon ligand binding
- Detectable regions:
  - Peptide bond (220 nm)
  - **Aromatic amino acids** (230-300 nm)
  - Metal ions, prosthetic groups (visible region)



DOI: 10.1039/C5RA13575C (Paper) *RSC Adv.*, 2015, **5**, 81533-81545

Spectroscopic exploration of drug–protein interaction: a study highlighting the dependence of the magnetic field effect on inter-radical separation distance formed during photoinduced electron transfer

Brotati Chakraborty[*a], **Piyali Mitra**[b] **and Samita Basu**[*b]



› J Pharm Sci. 2012 Sep;101(9):3051-61. doi: 10.1002/jps.23188. Epub 2012 May 11.

An application of ultraviolet spectroscopy to study interactions in proteins solutions at high concentrations

Santosh V Thakkar [1], Kevin M Allegre, Sangeeta B Joshi, David B Volkin, C Russell Middaugh

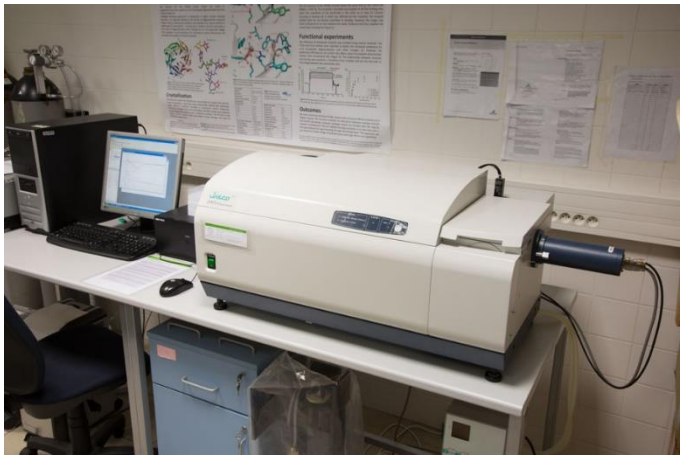
Affiliations + expand

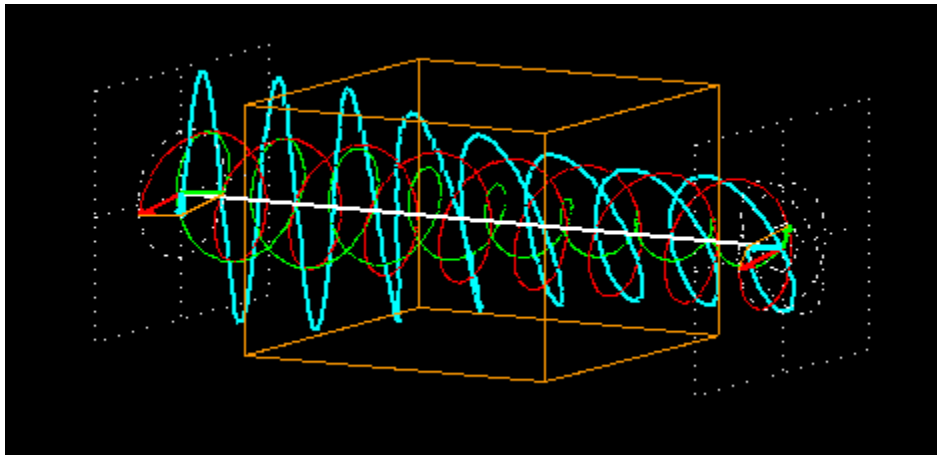
PMID: 22581726 DOI: 10.1002/jps.23188

Circular dichroism spectroscopy (CD)

- **Circular dichroism** – difference in absorption of left and right circularly polarized light by chiral compounds
- Binding can cause or affect CD of interacting partners
- Protein or NA may change 2D structure upon ligand binding

Circular dichroism $CD = \Delta A = A_L - A_R$ $\Delta\varepsilon = \varepsilon_L - \varepsilon_R = \frac{A_L - A_R}{c\ l}$ $3298.\Delta\varepsilon = [\theta]$ Molar elipticity

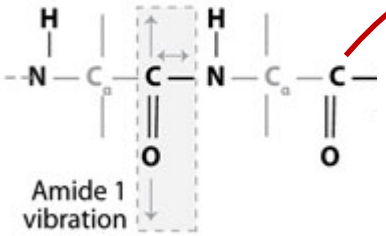


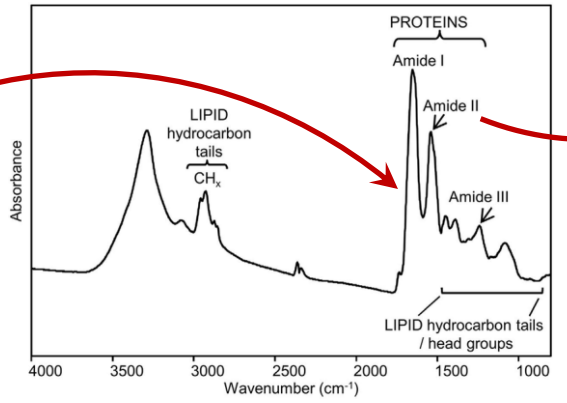


cddemo.szialab.org

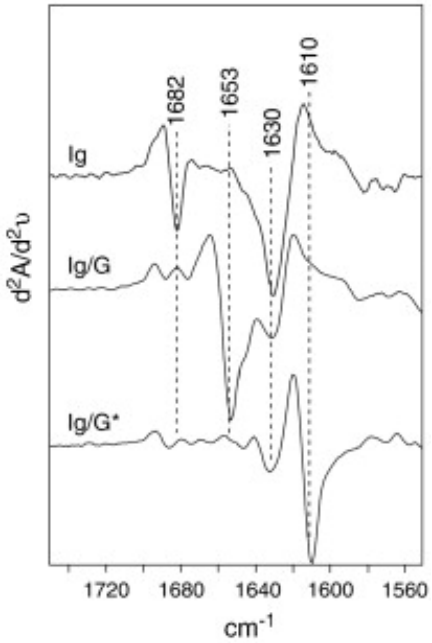
Fourier-transformed infrared spectroscopy (FTIR)

- Absorption of infrared light by various types of bonds (for protein C=O)
- Sensitive to protein secondary structure
- Applicable for **large interacting partners** (protein-protein, protein-membrane)
  - Usually require one protein to be isotopically labeled
  - On-surface variant: AFT-FTIR (attenuated total reflection FTIR) – suitable for large particles



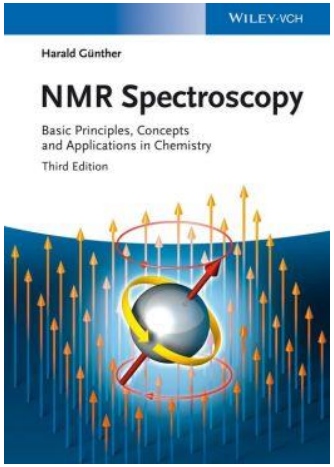


Ami et al, 2016

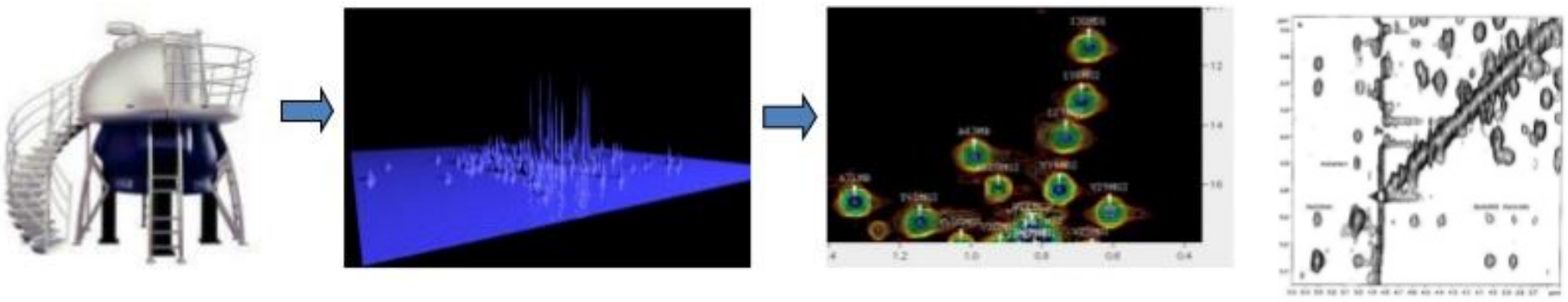


Li et al, 2009

Nuclear magnetic resonance (NMR) spectroscopy



- Based on interaction of **atom nuclei** with external **magnetic field**
- Ligand binding is detected as change in resonant frequency (chemical shift)
- Proteins are usually isotopically labeled (13C, 15N)
- Suitable especially for smaller molecules (< 50 kDa)



Nuclear magnetic resonance (NMR) spectroscopy

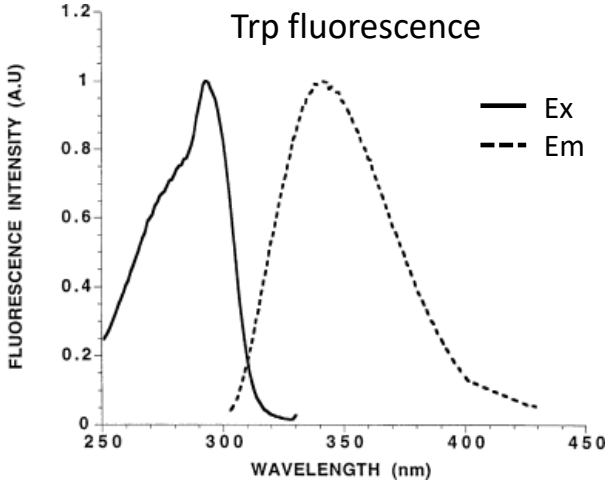
- **Advantages**
  - Sensitive to weak interactions
  - Large range of affinities and kinetics
  - Detailed view on the process (“atomic resolution”)
- **Disadvantages**
  - Concentrated labeled sample needed
  - Time consuming (no high throughput)
  - Expensive instrumentation (up to 10 mil €)

ceitec.eu

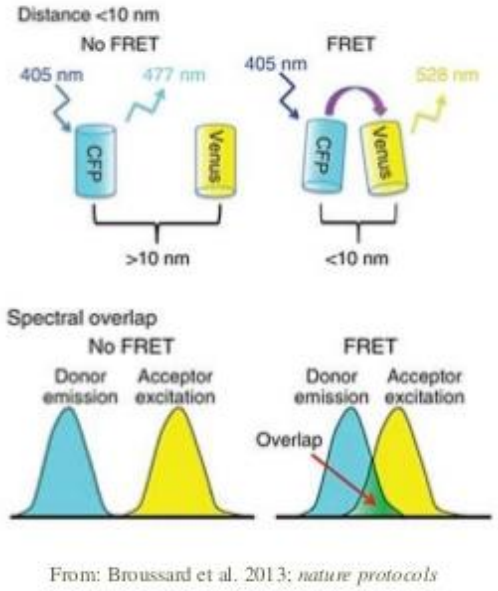
Fluorescence techniques

- **Fluorophores**
  - Intrinsic – Trp, Tyr, (Phe), GFP,...
  - Attached dyes – FITC, Alexa, DyLight,...
- **Features (Techniques)**
  - Fluorescence intensity
  - Wavelength shift – excitation, emission
  - Fluorescence anisotropy (polarization)
  - Fluorescence transfer – FRET
  - Time resolved fluorescence

More details in the previous lecture by Valentina Adami



Brancaleon et al. 1999

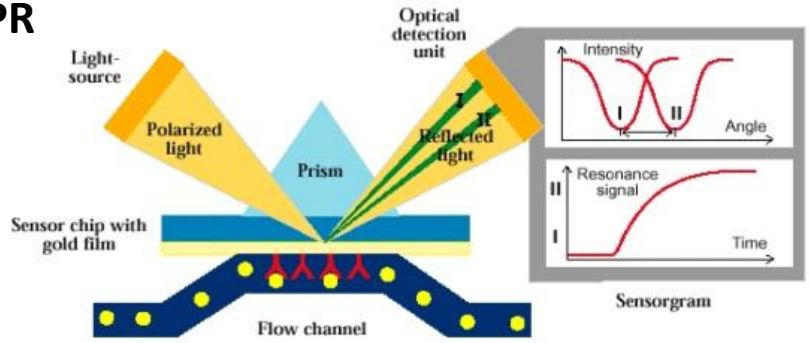


From: Broussard et al. 2013; *nature protocols*

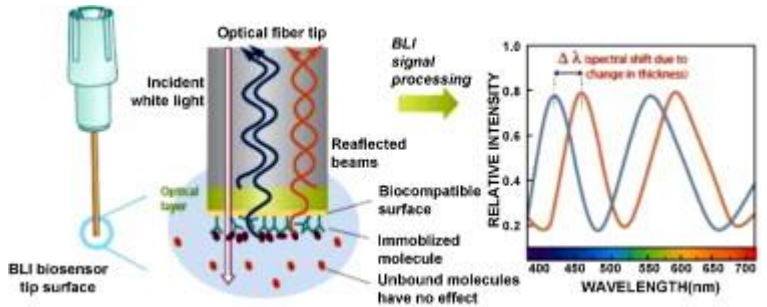
Optical biosensors

- Various principles:
  - **Surface plasmon resonance (SPR)**
  - **Bio-layer interferometry (BLI)**
- Detection of molecular interaction on a chip **surface**
- Require immobilization of one interacting partner
- Affinity, kinetics, inhibition

SPR



BLI



Wallner et al, 2013

Mobility-based methods

Mass spectrometry (MS)

- Protein complexes can be analyzed by ESI-MS
- Experiment provides directly stoichiometry of binding
- Other information can be obtained: relative affinity, interacting region, structural changes
- Hydrogen-deuterium exchange (HDX-MS) sometimes applied

Review > Methods Mol Biol. 2006;316:179-97. doi: 10.1385/1-59259-964-8:179.

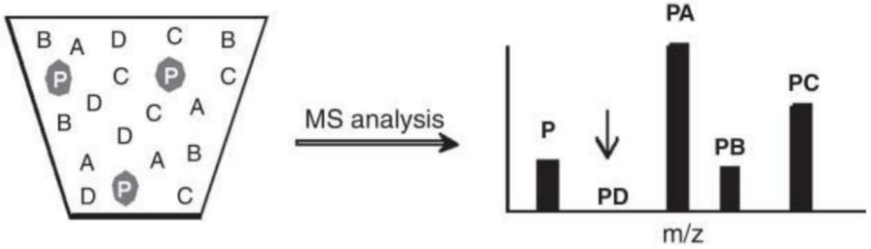
Protein interactions probed with mass spectrometry

Suma Kaveti [1], John R Engen

Affiliations + expand

PMID: 16671405 DOI: 10.1385/1-59259-964-8:179

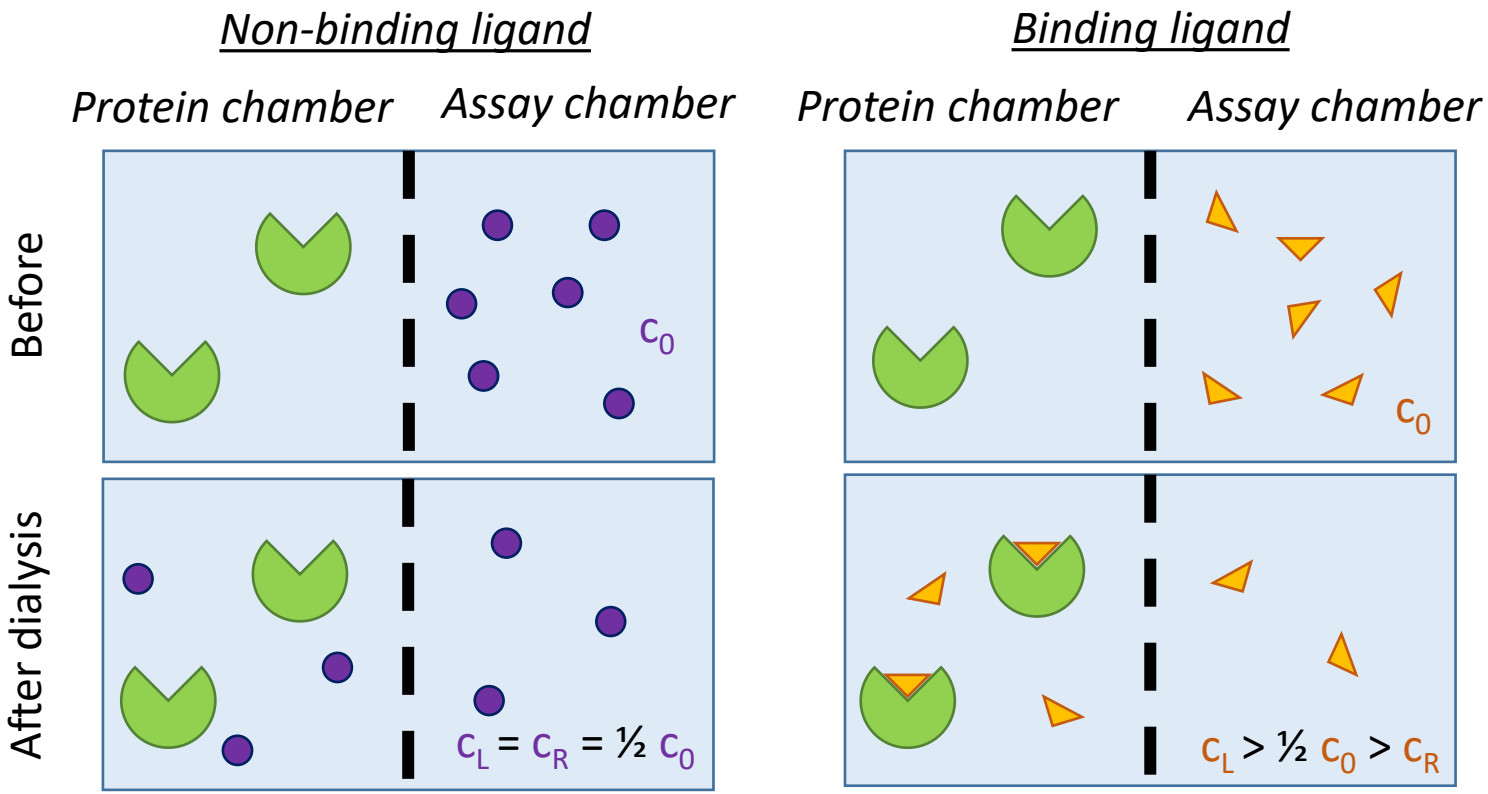
Affinity to ligands: A > C > B >>D



Equilibrium dialysis

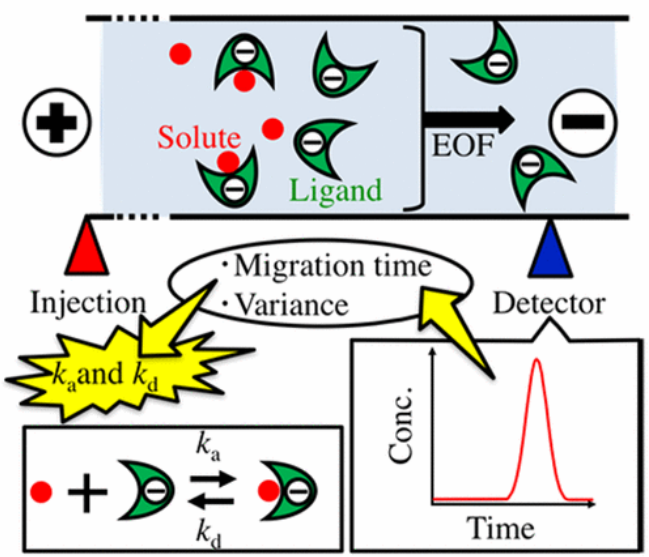
Thermo scientific

- Analysis of change in concentration after dialysis through **semi-permeable membrane**
- Set of experiments leads to affinity and stoichiometry

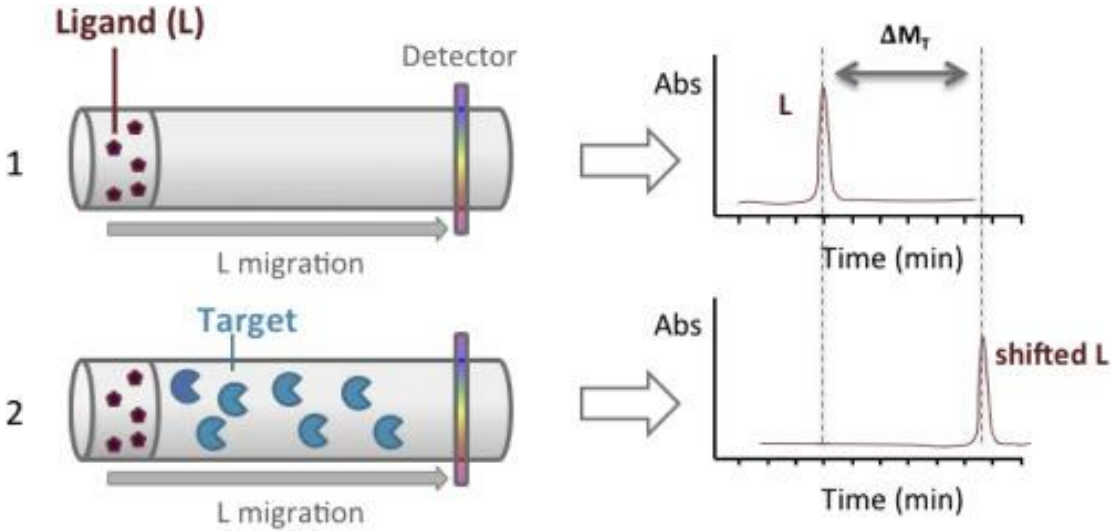


Affinity capillary electrophoresis (ACE)

- Electrophoresis in capillary
- Several variants: zone CE, gel CE, isotachophoresis,...
- Change in mobility of target or ligand upon formation of complex



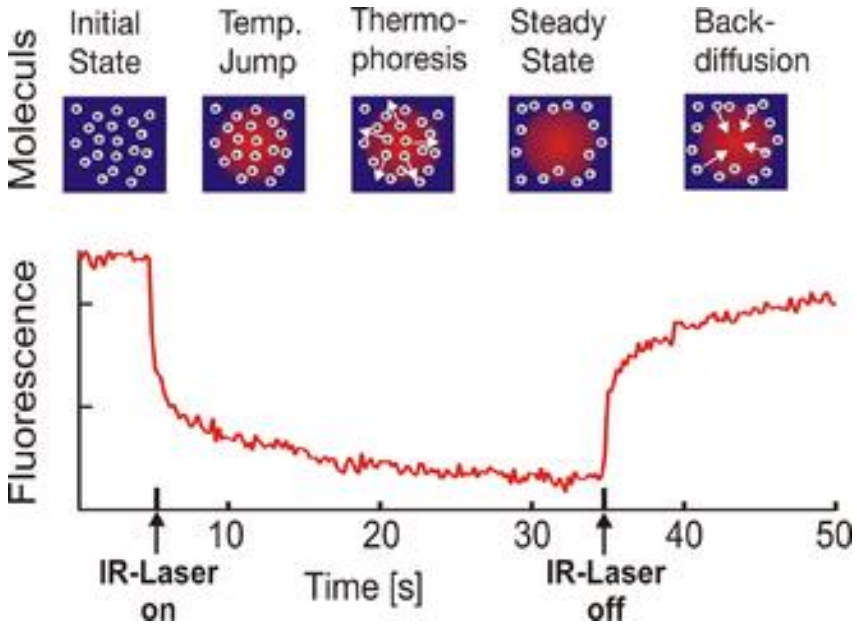
Suzuki & Miyabe, 2017

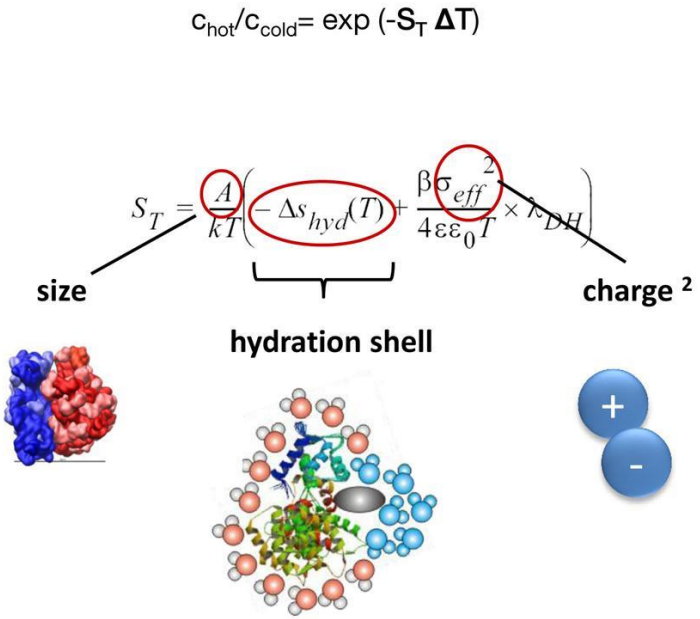


Farcas et al, 2017

Micro-scale thermophoresis (MST)

- Motion of molecules in microscopic **temperature gradient**
- Sensitive to hydration, charge, size – detection of ligand binding
- Detection via fluorescence (intrinsic or attached dye)

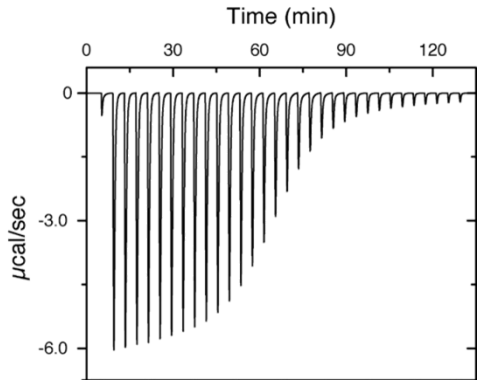




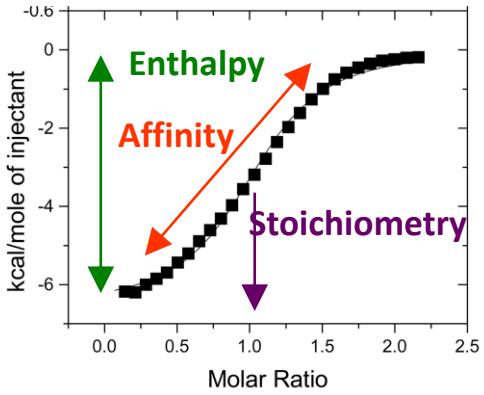
Calorimetrical methods

Isothermal titration calorimetry (ITC)

- All processes (including protein-ligand interaction) are associated with heat absorption or release
- ITC detects **heat change** caused by ligand binding
- Measurement in solution, no labeling
- Determines: affinity (K_D/K_A), thermodynamics (ΔH, ΔG, ΔS), stoichiometry (n)

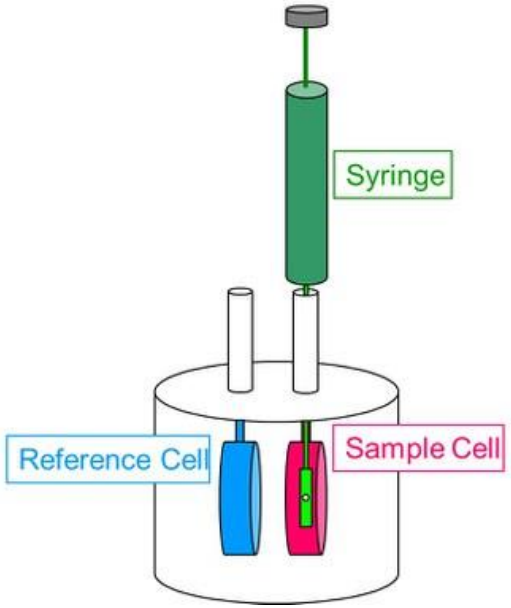


$$P + L \underset{K_D}{\overset{K_A}{\rightleftharpoons}} PL$$



$$\Delta G^\circ = -RT \ln K_A$$

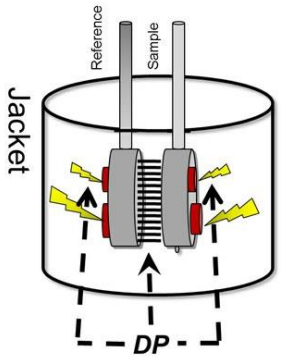
$$\Delta G^\circ = \Delta H^\circ - T\Delta S^\circ$$



Stability-based methods

Differential scanning calorimetry (DSC)

- Measures changes in heat over a range of temperatures
- Interaction with ligand stabilize (mostly) the protein –> higher protein T_m
- Stabilization is given by **ligand binding energy** – can be quantified



Contents lists available at ScienceDirect

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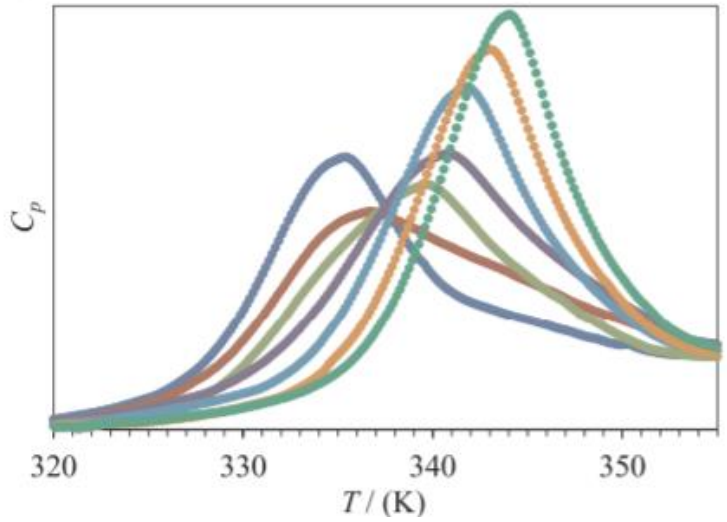
journal homepage: www.elsevier.com/locate/ijpharm

Evaluation of the binding properties of drugs to albumin from DSC thermograms

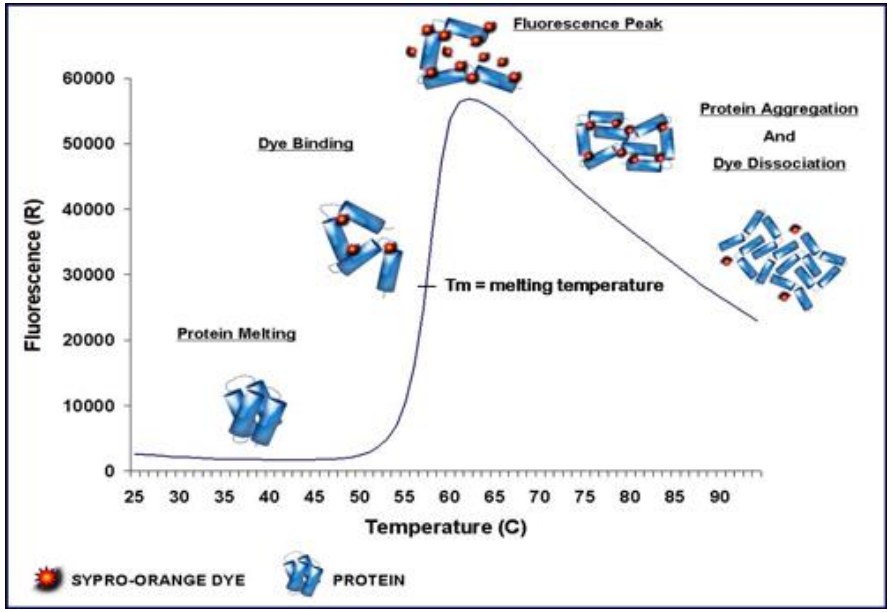
Igor Sedov*, Alena Nikiforova, Diliara Khaibrakhmanova

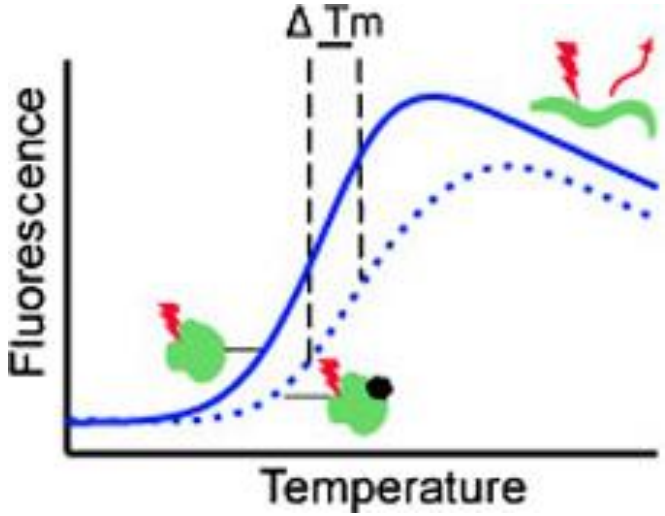
Chemical Institute, Kremlevskaya 18, Kazan Federal University, 420008 Kazan, Russia



Thermal shift assay (TSA)

- An increase in the **melting temperature** of the target protein in the presence of a test ligand is indicative of a promising ligand–protein interaction.
- High-throughput possibility





Structure based

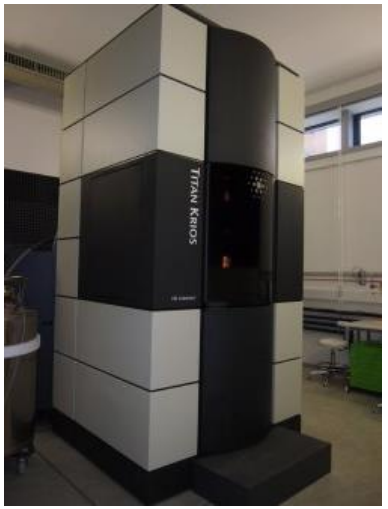
Structure-based techniques

- Atomic-level structure of a complex
- Technically demanding
- Detailed view on the binding

X-ray

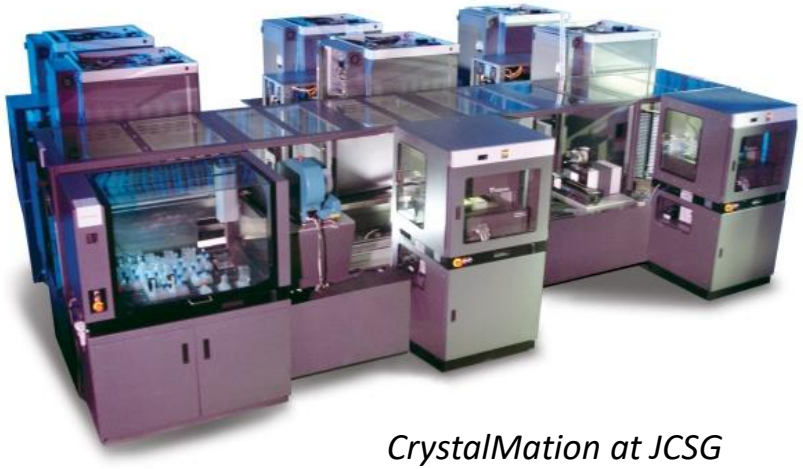
NMR

Cryo-EM

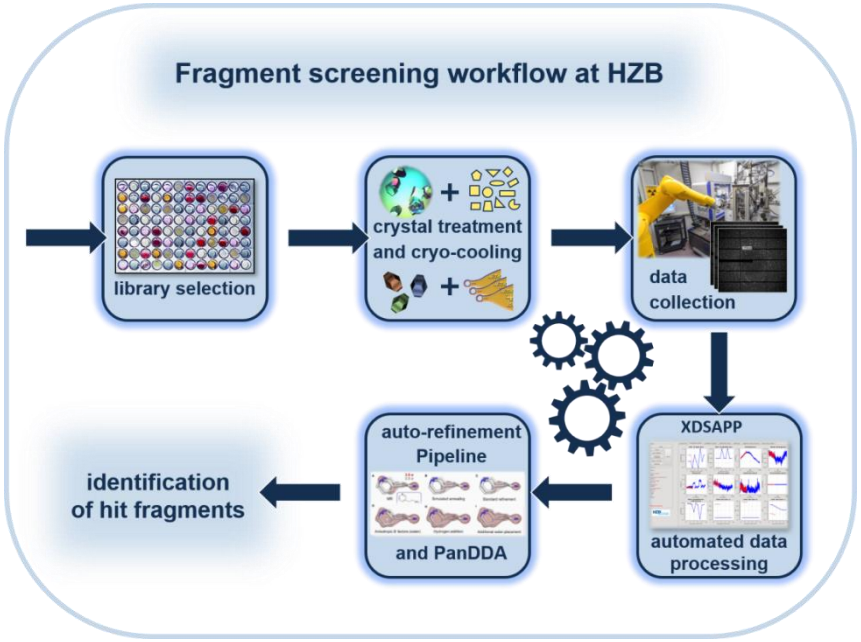


X-ray crystallography

- Currently the only structural technique with high-throughput possibility
- Usually long initial optimization (months)
- Applied for fragment screening



CrystalMation at JCSG (Blow 2008)



www.helmholtz-berlin.de

Other methods

Electrochemical methods

- Individual techniques target different electrochemical features (current, charge potential, conductivity)
- Important for **biosensors** development

Review > Mater Sci Eng C Mater Biol Appl. 2016 Apr 1;61:1002-17.
doi: 10.1016/j.msec.2015.12.020. Epub 2015 Dec 11.

Pharmacogenomic study using bio- and nanobioelectrochemistry: Drug–DNA interaction

Mohammad Hasanzadeh [1], Nasrin Shadjou [2]

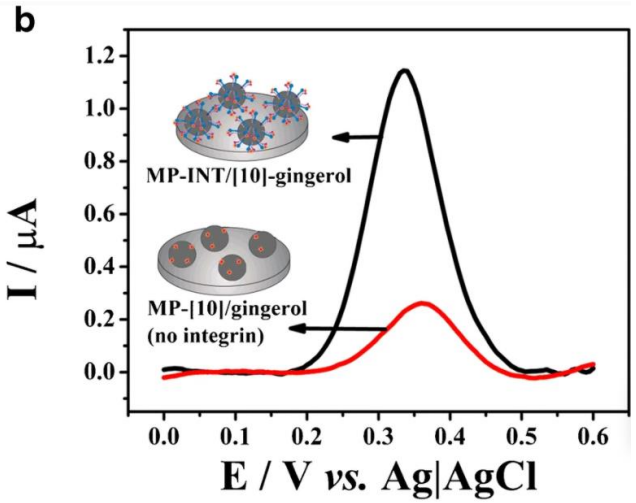
Affiliations + expand
PMID: 26838928 DOI: 10.1016/j.msec.2015.12.020

Review > Acta Chim Slov. 2014;61(3):555-73.

An overview of the optical and electrochemical methods for detection of DNA – drug interactions

Mara M Aleksić, Vera Kapetanović

PMID: 25286211



Uliana et al 2019

Complex techniques

- Indirect detection of molecular interaction
- **Multi-step approaches**
- Mainly used for protein-protein interactions
- Examples
  - Yeast two-hybrid
  - Phage display
  - Pull-down assay
  - Co-immunoprecipitation
  - …

Review > Curr Opin Chem Biol. 1999 Feb;3(1):64-70. doi: 10.1016/s1367-5931(99)80012-x.

Progress and variations in two-hybrid and three-hybrid technologies

B L Drees [1]

Affiliations + expand

PMID: 10021404 DOI: 10.1016/s1367-5931(99)80012-x

Review > Cell Mol Life Sci. 2010 Mar;67(5):749-67. doi: 10.1007/s00018-009-0192-2.

Progress in phage display: evolution of the technique and its application

Tomaz Bratkovic [1]

Affiliations + expand

PMID: 20196239 DOI: 10.1007/s00018-009-0192-2

Review > Methods. 2001 Jul;24(3):218-29. doi: 10.1006/meth.2001.1183.

The tandem affinity purification (TAP) method: a general procedure of protein complex purification

O Puig [1], F Caspary, G Rigaut, B Rutz, E Bouveret, E Bragado-Nilsson, M Wilm, B Séraphin

Affiliations + expand

PMID: 11403571 DOI: 10.1006/meth.2001.1183

> Methods Mol Biol. 2017;1615:211-219. doi: 10.1007/978-1-4939-7033-9_17.

Protein-Protein Interactions: Co-Immunoprecipitation

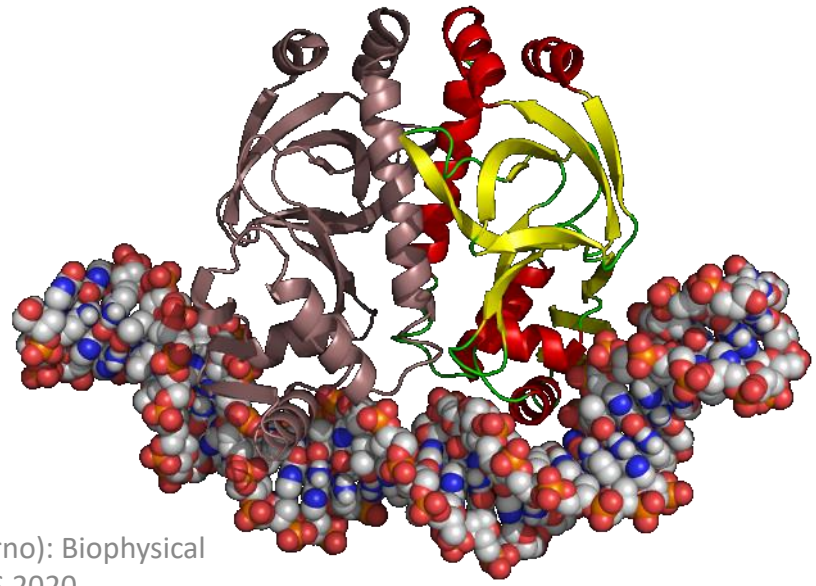
Jer-Sheng Lin [1], Erh-Min Lai [2]

Affiliations + expand

PMID: 28667615 DOI: 10.1007/978-1-4939-7033-9_17

Protein-Nucleic acid interaction

- DNA-binding proteins constitute 10% of protein-coding genes in eukaryotes
- They interact both in **specific** (e.g. transcription factor) and **non-specific** (e.g. histones) way
  = sequence dependent / independent
- Various techniques
  - Similar to protein-ligand
  - Specific



Protein-DNA interaction detection

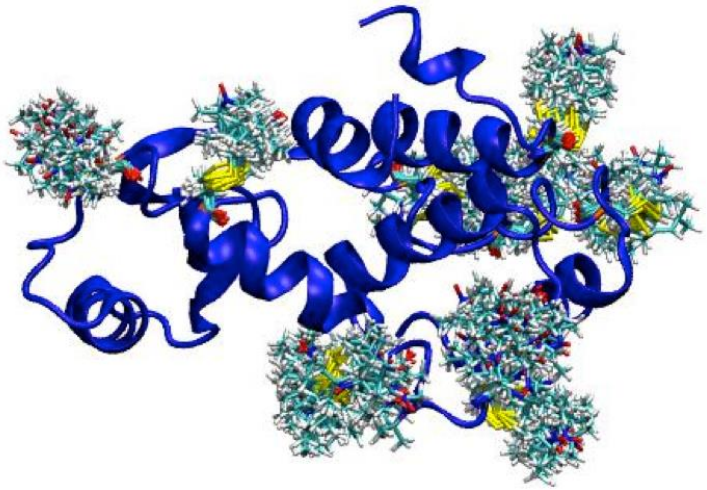
- Chromatin immunoprecipitation
- DNA electrophoretic mobility shift assay (EMSA)
- DNA pull-down assay
- Reporter assay
- Microplate capture
- DNA footprinting

Selected References for Studying Protein-DNA Interactions:

1. Evertts A.G., *et al.* (2010). Modern approaches for investigating epigenetic signaling pathways. *J Appl Physiol.* Jan 28. [Epub ahead of print]
2. Georges, A.B., *et al.* (2010). Generic binding sites, deneric DNA-binding domains: Where does specific promoter recognition come from? *FASEB Journal*,**24**: 346-356.
3. Griffiths, Anthony J. F., *et al.*, eds (2000). "Genetics and the Organism: Introduction". An Introduction to Genetic Analysis (7th ed.). New York: W. H. Freeman.
4. Halford, S.E. and Marko, J. (2004). How do site specific DNA-binding proteins find their target? *Nuc. Acid Research.* **32(10):** 3040-3052.
5. Hartl, Daniel L., *et al.* (1988). Basic Genetics, Boston: Jones and Bartlett Publishers, Inc.
6. Kress, C., *et al.* (2010). Epigenetic modifications in 3D: Nuclear organization of the differentiating mammary epithelial cell. *J Mammary Gland Biol Neoplasia.*Feb 10. [Epub ahead of print]

Computational methods

- Molecular docking
- Virtual screening
- Molecular dynamics
- Database search



- ➢ Relatively cheap
- ➢ Less accurate
- ➢ Ideally to be combined with experimental approaches

Break