Biomolecular interactions - Introduction

S2004

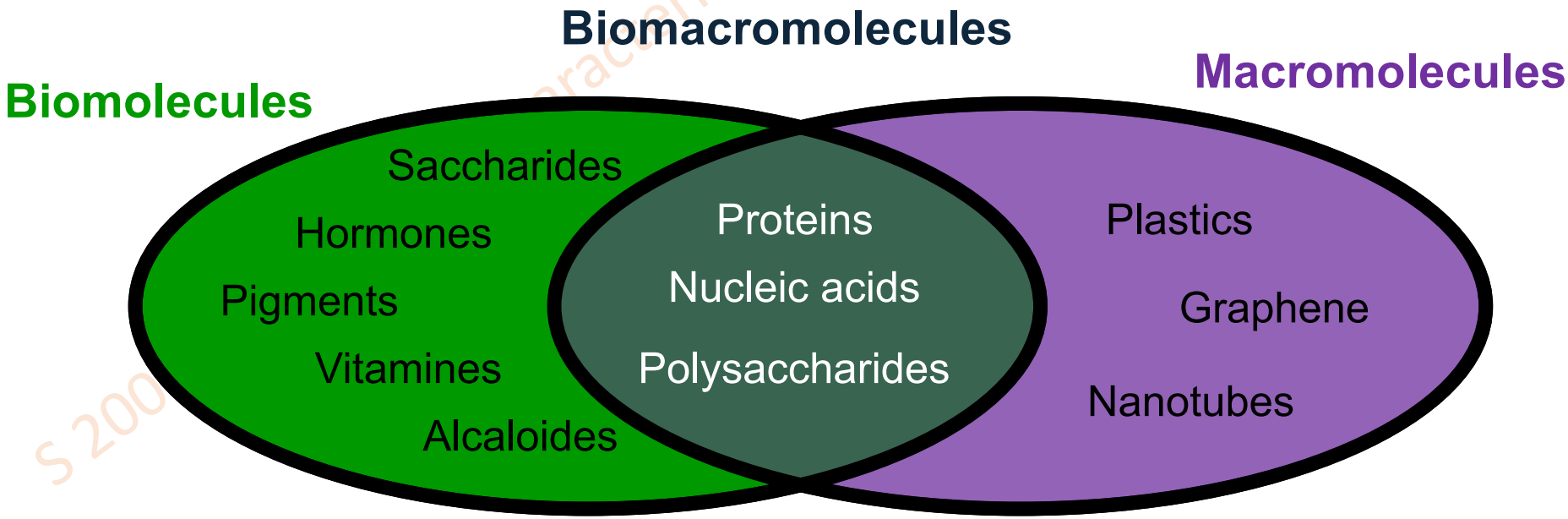
Methods for characterization of biomolecular interactions – classical versus modern

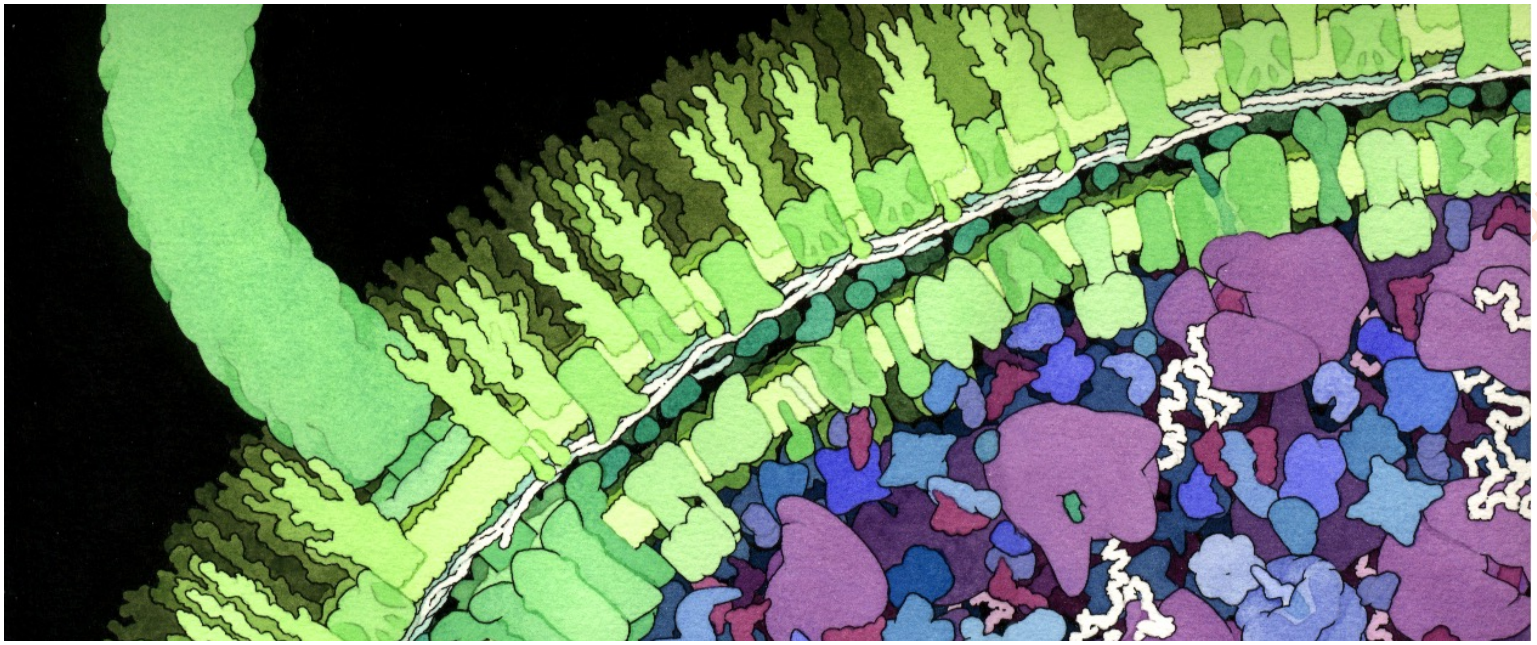
Biomacromolecules

Biomolecules are naturally present in living organisms.

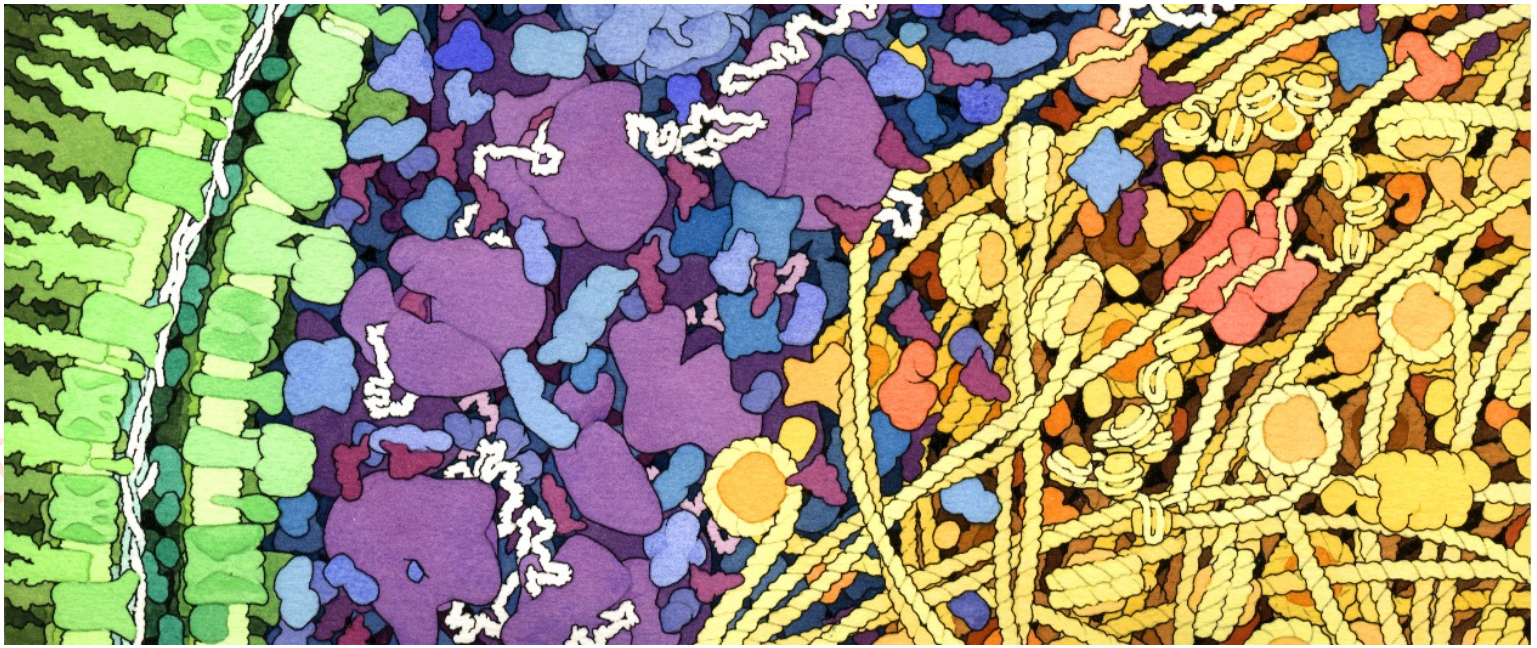
Macromolecules. While small molecules consist of up to several hundreds of atoms, macromolecules consist of thousands to millions of atoms.

Molecules are basic blocks of matter. They are formed by atoms linked through covalent bonds.





Molecular interactions are central to the molecular basis of life



Biomolecular interactions are everywhere…

Protein – Ligand

Protein – Protein

Protein – Nucleic acid

Nucleic acid – Ligand

Protein/NA adsorption

Protein – Solvent

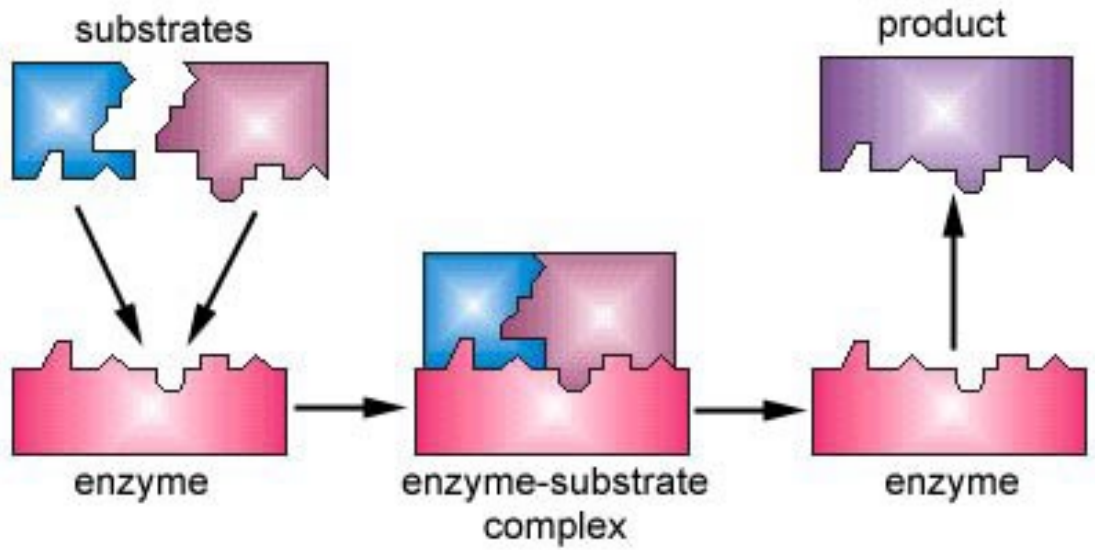
Nucleic acid – Solvent

Protein – Inorganic salt

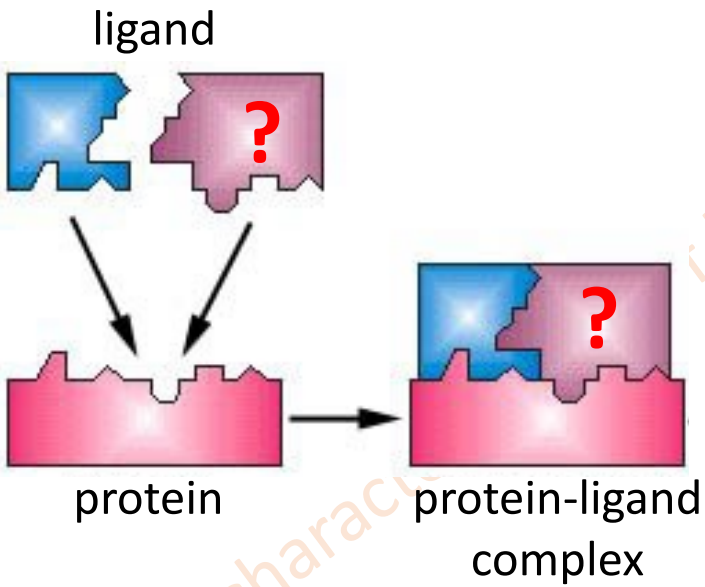
Nucleic acid – Inorganic salt

All processes in living organisms are essentially determined by biomolecular interactions

Interaction vs. chemical reaction



Interaction vs. chemical reaction



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

Types of interaction

- **Nuclear physics**

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

- **Chemistry** (electron ionization)

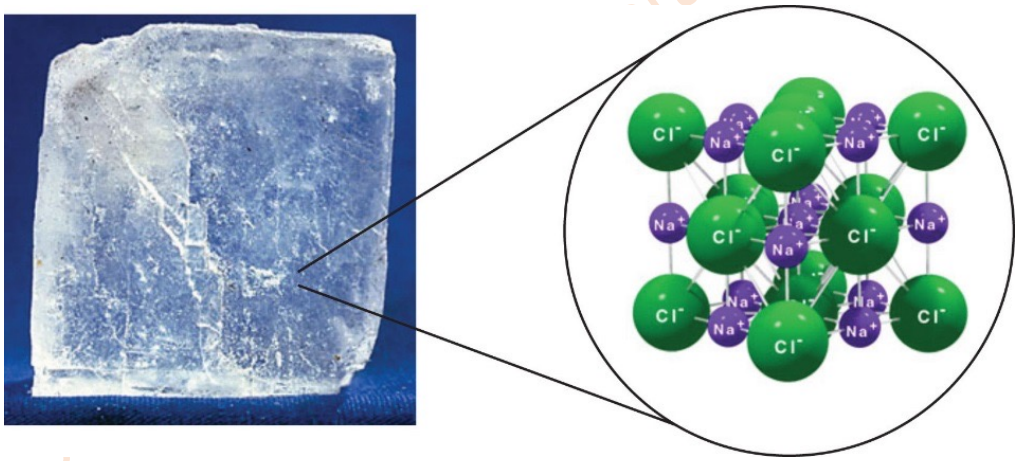
  formation of bonds 150-1000 kJ/mol

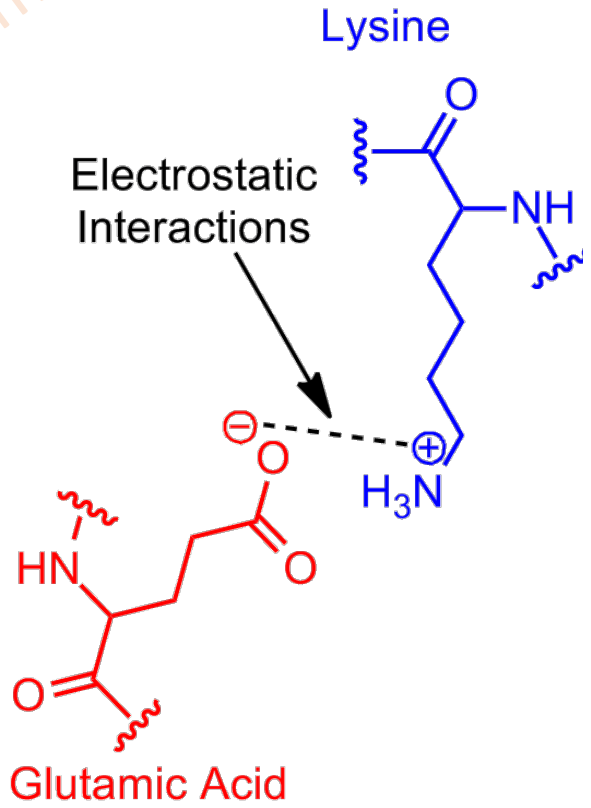
- **Biochemistry-biology**

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

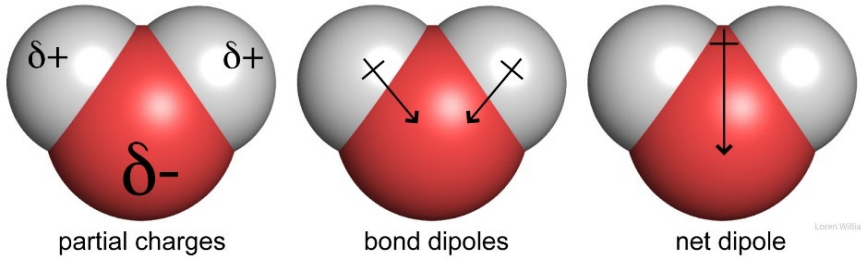
Coulombic interactions (salt bridge)

- Charged atoms = ions
- Same charge – repulsion
- Opposite charge – **attraction**

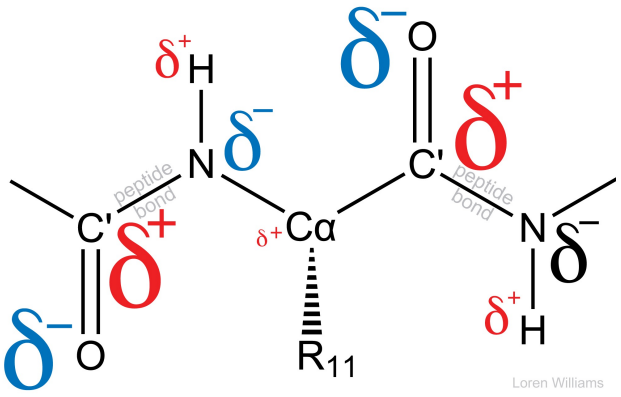


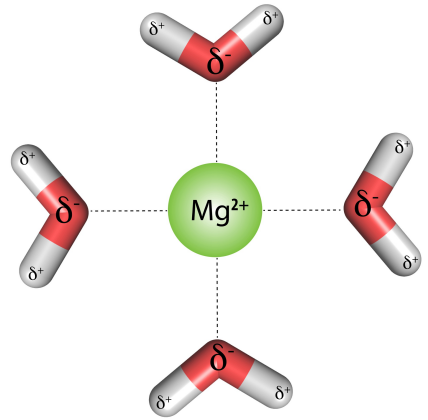


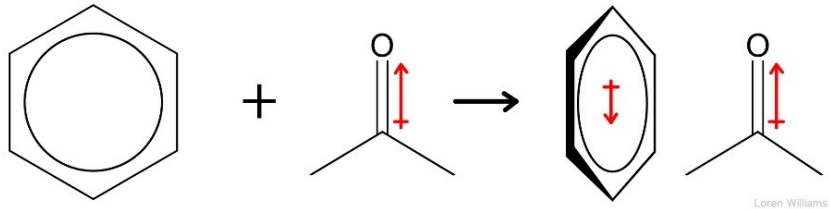
Dipole interactions



- **Dipole** – unequal distribution of electrons in molecule – orientation-dependant
- Dipole-dipole, dipole-charge, dipole-induced dipole

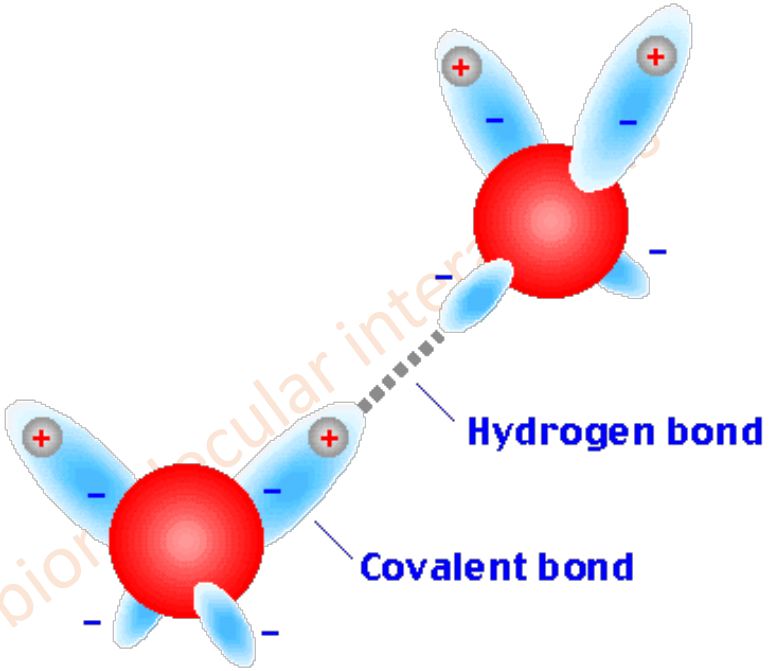


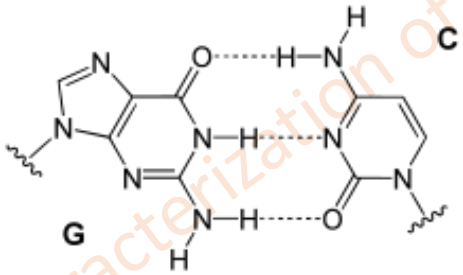




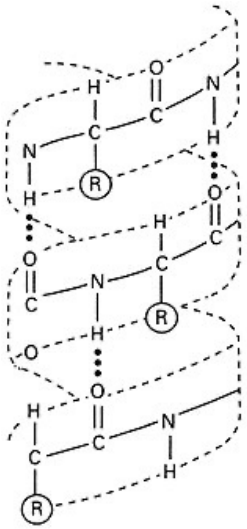
Hydrogen bonds

- Atom with free electron pair + hydrogen bound to electronegative atom (O, N, x, s, c, …)

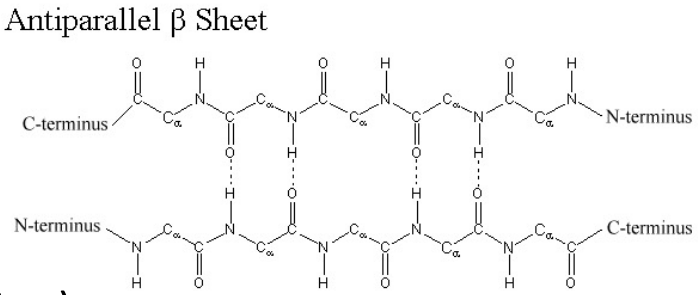


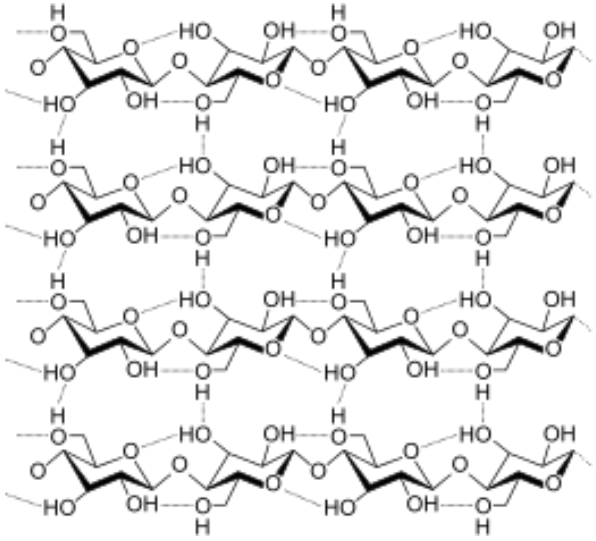


DNA (base pairing)



Protein
(2D structure stabilization)



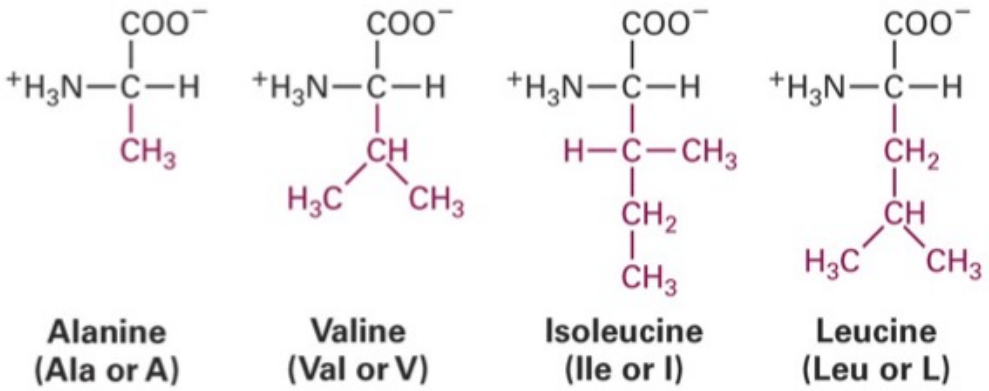


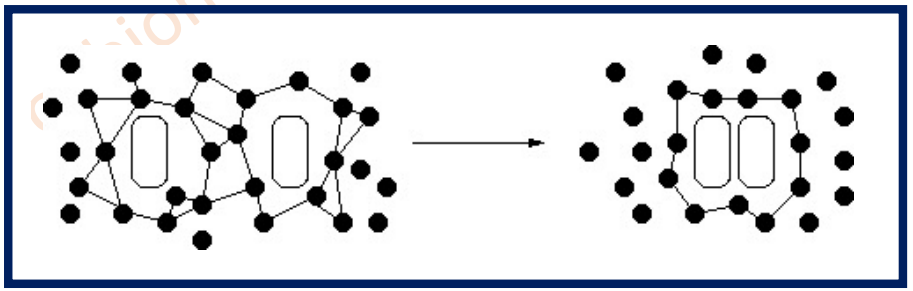
Polysaccharide (cellulose)

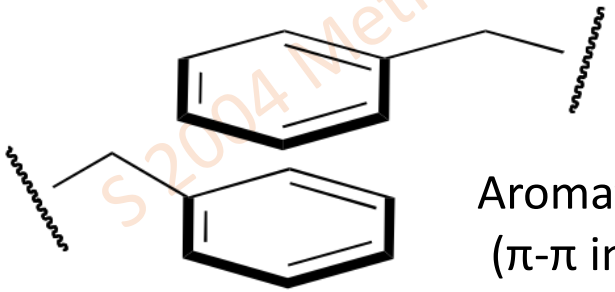
Hydrophobic interactions

(van der Waals, nonpolar interactions)

- Driven by entropy – strong influence of temperature

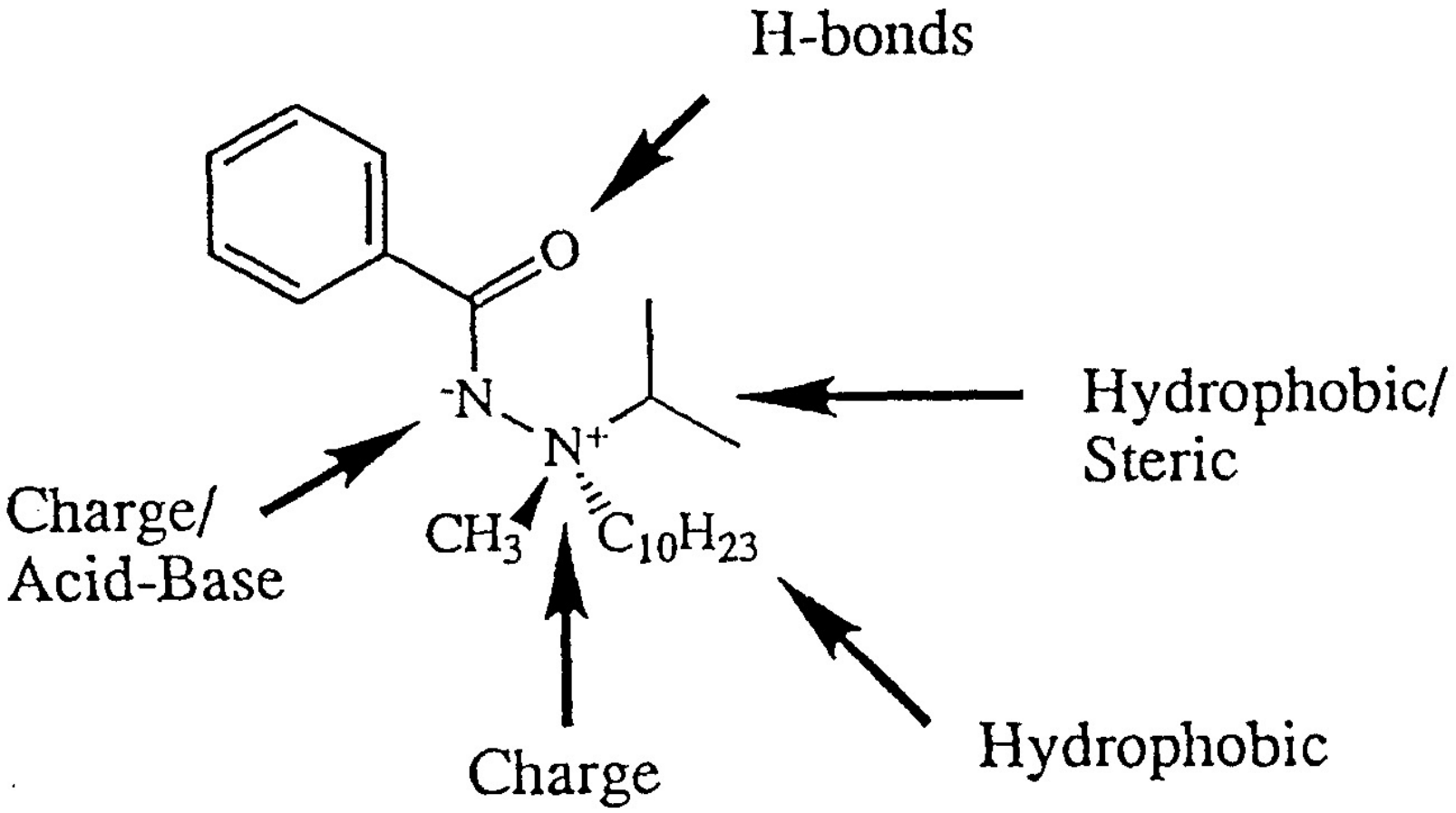




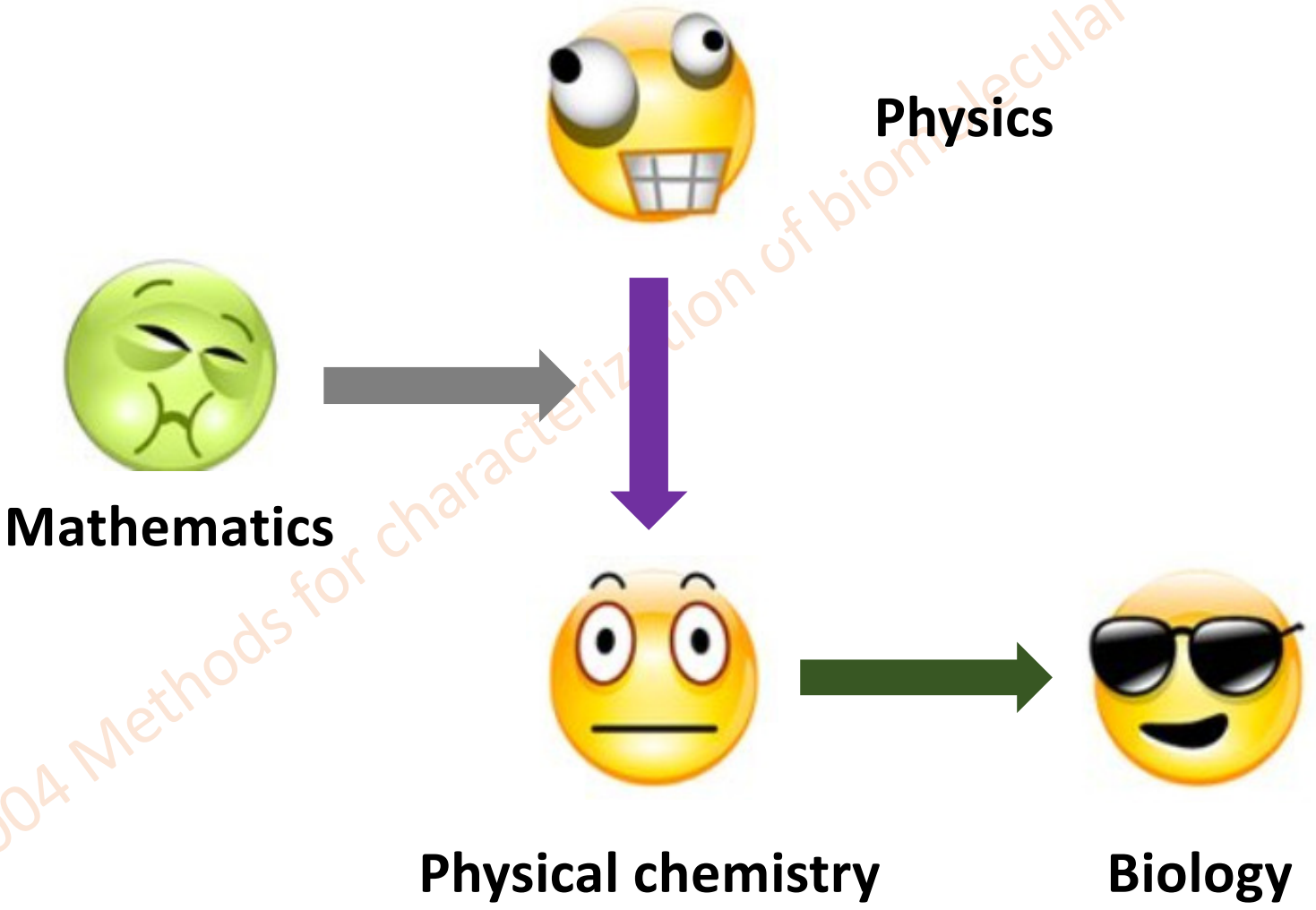


Aromatic stacking
(π-π interaction)

Mostly more than one effect is present



Interaction description



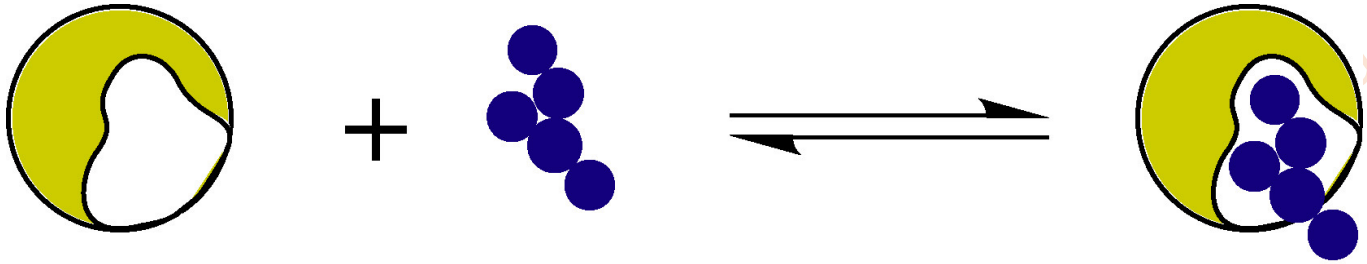
Why to study the interactions

- **Understanding of biological processes**
  - Does it bind?
  - How strong the interaction is?
  - 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

Receptor – ligand interaction



M X MX

$$K_A = \frac{[MX]}{[M][X]}$$

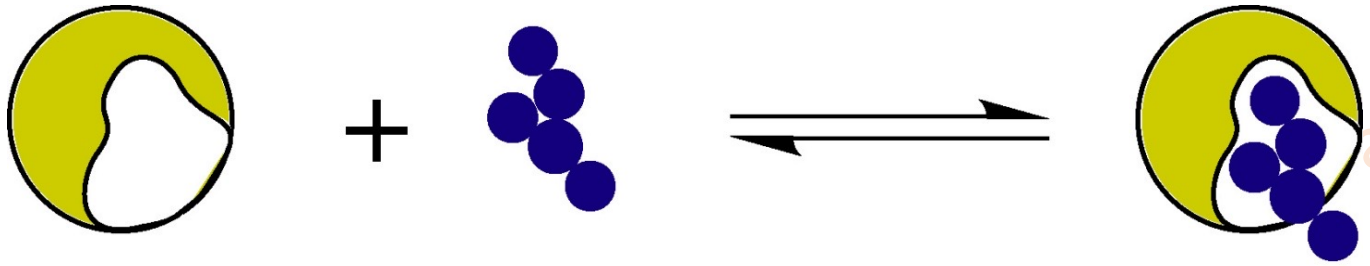
Units: L / mol (M^{-1})

$$K_D = \frac{[M][X]}{[MX]}$$

Units: mol / L (M)
i.e. K_d is a concentration

High affinity = large K_a, small K_d

Receptor – ligand interaction



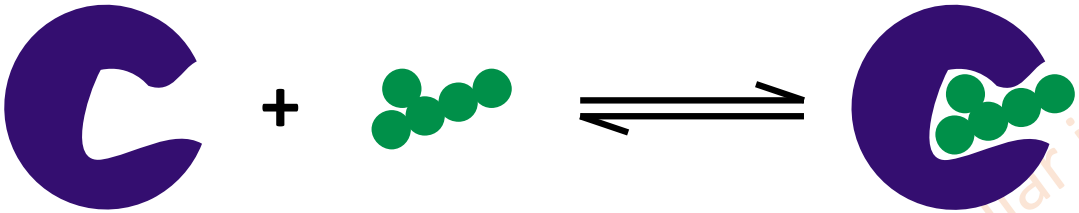
M **X** **MX**

$$association: \frac{d[\mathrm{MX}]}{dt} = k_a[\mathrm{M}][\mathrm{X}]$$ Units: $M^{-1}s^{-1}$

$$dissociation: \frac{d[\mathrm{MX}]}{dt} = k_d[\mathrm{MX}]$$ Units: s^{-1}

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

Receptor – ligand interaction

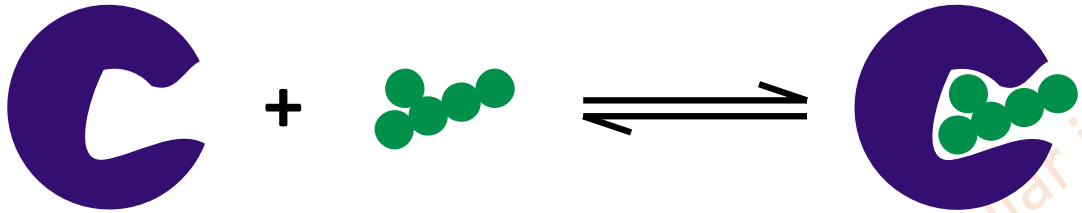


$$\frac{d[\mathrm{MX}]}{dt} = k_a[\mathrm{M}][\mathrm{X}] - k_d[\mathrm{MX}]$$

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

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

Gibbs energy, enthalpy, entropy



$$M + X \underset{K_D}{\overset{K_A}{\rightleftharpoons}} MX$$

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

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

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

High affinity = large K_a, small K_d, large $-\Delta G^\circ$

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

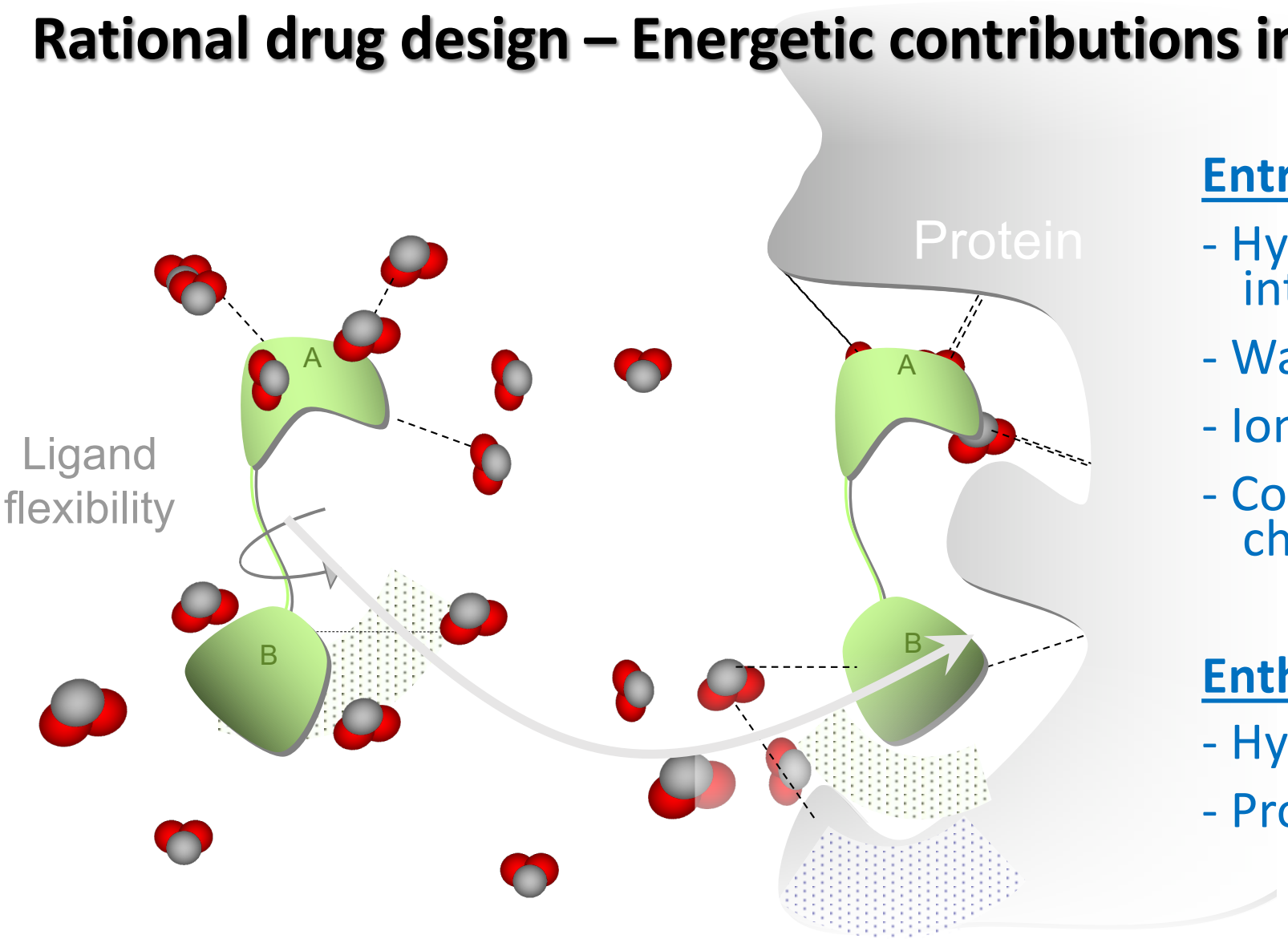
- flexible molecules lose the entropy upon binding

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

Hydrogen bonds

Hydrophobic parts

Water molecules

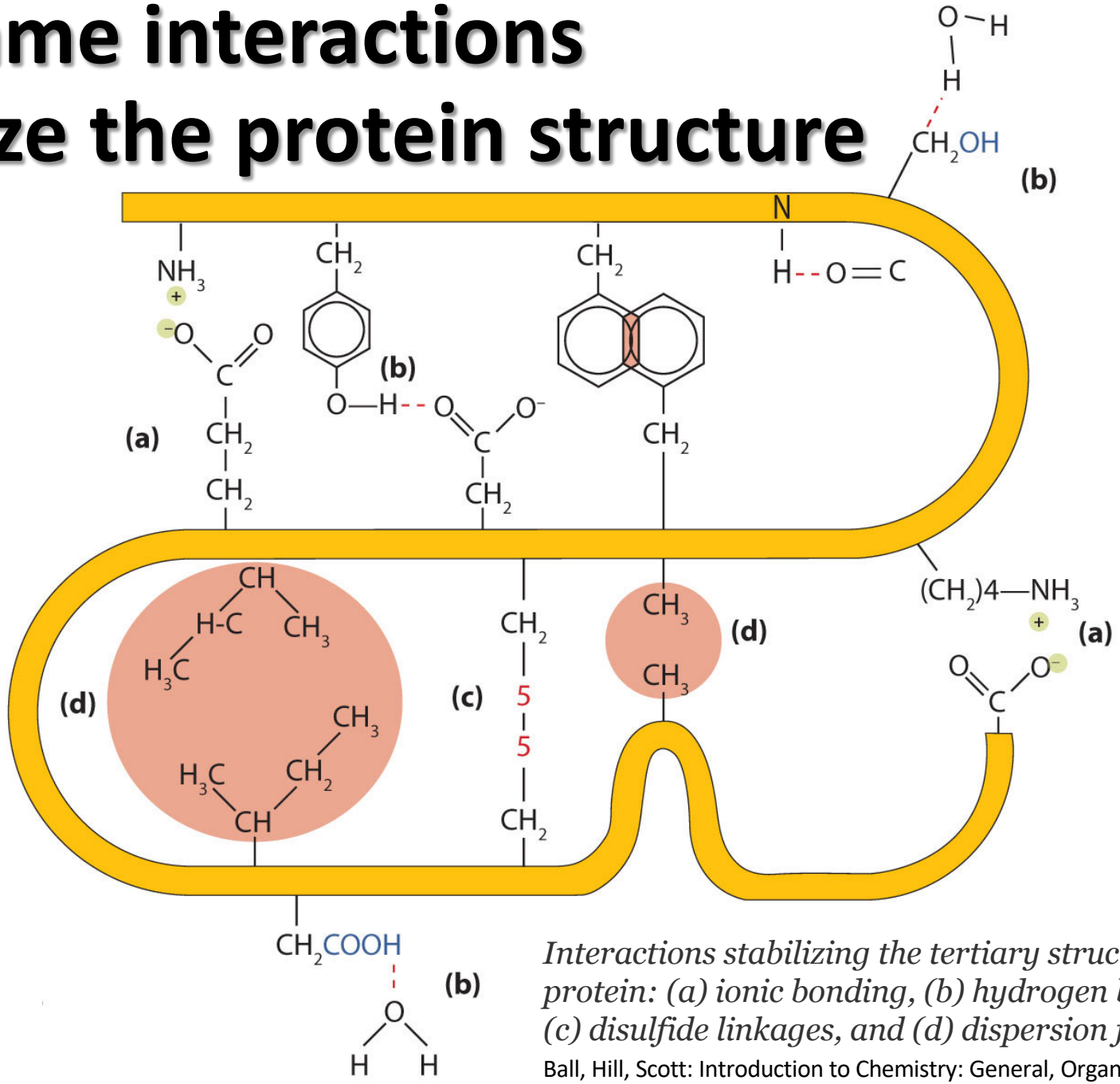
Rational drug design – Energetic contributions involved

Why kinetics of interaction is important?

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

$$1\ \text{nM} = \frac{10^{-2}\ s^{-1}}{10^{7}\ M^{-1}s^{-1}} = \frac{10^{-5}\ s^{-1}}{10^{4}\ M^{-1}s^{-1}}$$

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

Methods to study interaction between molecules

- Optical and spectroscopic methods
  X-ray, CD, NMR, Mass spektrometry,, fluorescence, IR, Raman….
- Hydrodynamics
  sedimentation, ekvillibrium dialysis, capillary electrophoresis, …
- Direct measurements
  AFM, SPR, BLI, piezoelectric biosensors, immunochemistry, microarray…
- Calorimetry
- Molecular modelling
- …

Experimental techniques to measure the interactions

- Physical background
- Speed of analysis
- Suitable system studied

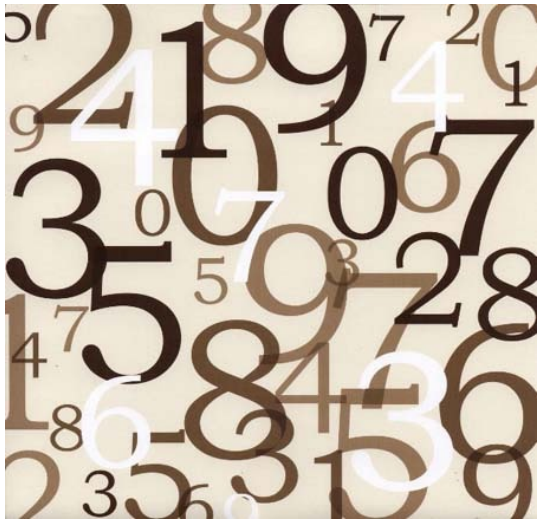
- Availability
- Complementarity
- “Fashion”

Two informational levels of methods

Qualitative

Semi-quantitative

Quantitative



Ultra violet-visible spectroscopy (UV-Vis)

- Absorption spectroscopy in the visible and ultraviolet spectral regions is a powerful technique by which ligand binding equilibria can be studied.

Three different internal chromophores in Biomolecule

- **The peptide bond** (absorbs weakly 220 nm)
- **Aromatic amino acids** F, Y, W, H (230–300 nm)
- **Many biological molecules** show strong absorbance in the visible region of the spectrum as a result of the presence of metal ions and prosthetic groups with extended π-electron systems, such as chlorophyll, carotinoid, flavin, heme.

These bands are sensitive to the surrounding polypeptide environment and reflect structural changes, oxidation states, and the binding of ligands.

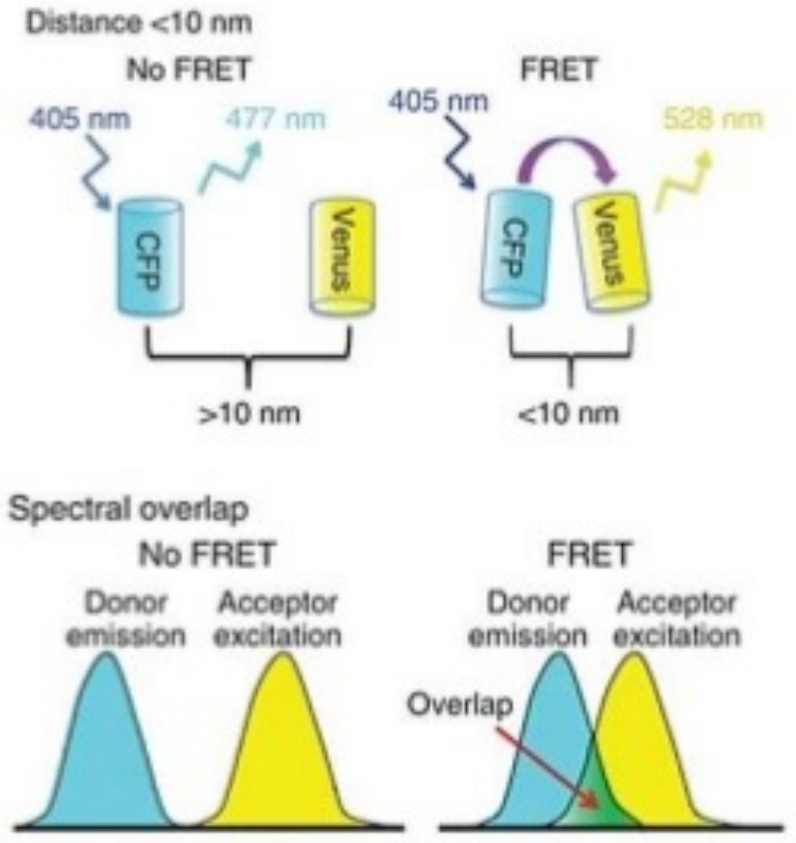
Nienhaus, Karin, and G. Ulrich Nienhaus. "Probing heme protein-ligand interactions by UV/visible absorption spectroscopy." *Protein-Ligand Interactions*. Humana Press, 2005. 215-241.

Fluorescence Resonance Energy Transfer (FRET)

- Donor and acceptor molecules must be in close proximity (10–100 Å).

- The absorption spectrum of the acceptor must overlap the fluorescence emission spectrum of the donor.

- The donor absorption and emission spectra should have a minimal overlap to reduce self-transfer.

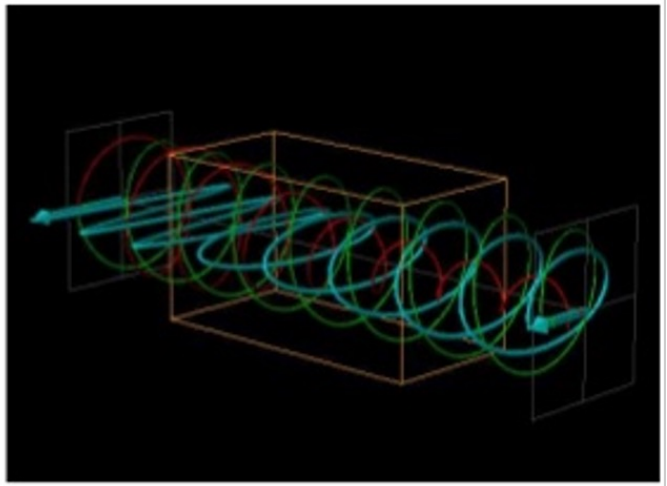


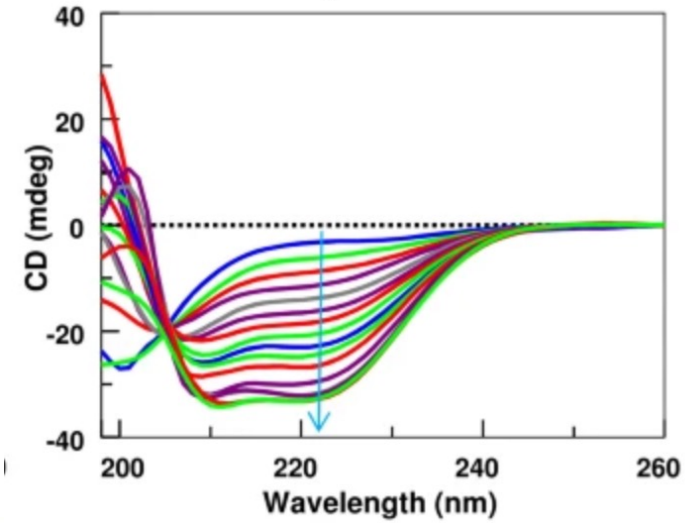
From: Broussard et al. 2013; *nature protocols*

Song, Yang, et al. "Protein interaction affinity determination by quantitative FRET technology." *Biotechnology and bioengineering* 109.11 (2012): 2875-2883.

Circular dichroism spectroscopy (CD)

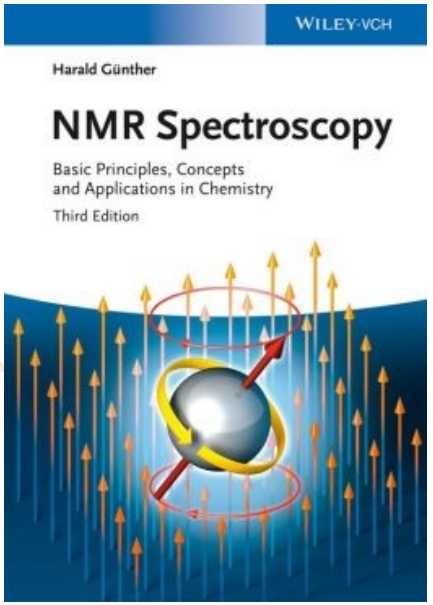
- CD is the difference in absorption of left and right circularly polarized light.
- Proteins and DNA and many ligands are chiral.
- Molecular interactions between chiral and achiral compounds can give rise to induced circular dichroism (ICD) of the achiral counterpart.
- If it is chiral then its ICD is the difference between its own CD spectrum and the spectrum in the presence of the protein.





Rodger, Alison, et al. "Circular dichroism spectroscopy for the study of protein-ligand interactions." *Protein-Ligand Interactions*. Humana Press, 2005. 343-363.

Nuclear magnetic resonance (NMR) spectroscopy



- A physical phenomenon in which nuclei in a magnetic field absorb and re-emit electromagnetic radiation.
- NMR detects ligand binding through changes in the resonant frequencies (chemical shifts) of NMR-active nuclei.
- NMR spectroscopy detects and reveals protein ligand interactions with a large range of affinities (10^{9}–10^{-3} M).
- Protein samples need to be isotopically enriched (15N and/or 13C).
- Larger molecules (>25 kDa), additional enrichment with 2H.
- Isotopically labeled protein → over-express in bacteria grown in minimal media containing 15NH4Cl and/or 13C glucose as the sole sources of nitrogen and/or carbon.

Mittermaier, Anthony, and Erick Meneses. "Analyzing Protein–Ligand Interactions by Dynamic NMR Spectroscopy." *Protein-Ligand Interactions*. Humana Press, 2013. 243-266.

Advantage	Drawback
Very sensitive to weak interactions	Needs concentrated isotopically lablled sample 50µM- 2mM)
Reveals the portion of molecule involved in interaction	Not suitable for >100 KDa
Accurate kinetics even for short lifetime bounds (< 1ms)	Needs high purity sample
Assay in equilibrium solution	Requires ligand-receptor buffer harmony
Quantitative (large range of affinities)	Strong magnetic fields needed for high quality → expensive
-	Long assay time

Saturation transfer difference (STD) - NMR

- powerful method for studying protein-ligand interactions in solution
- identifies the binding epitope of a ligand when bound to its receptor protein
- Ligand protons that are in close contact with the receptor protein receive a higher degree of saturation in contrast to protons that are either less or not involved in the binding process.
- applicable dissociation constants: 10^{-3}-10^{-8} M
- Irradiating the spectral region of broad resonances of the macromolecule which is free of any smaller molecule signals.

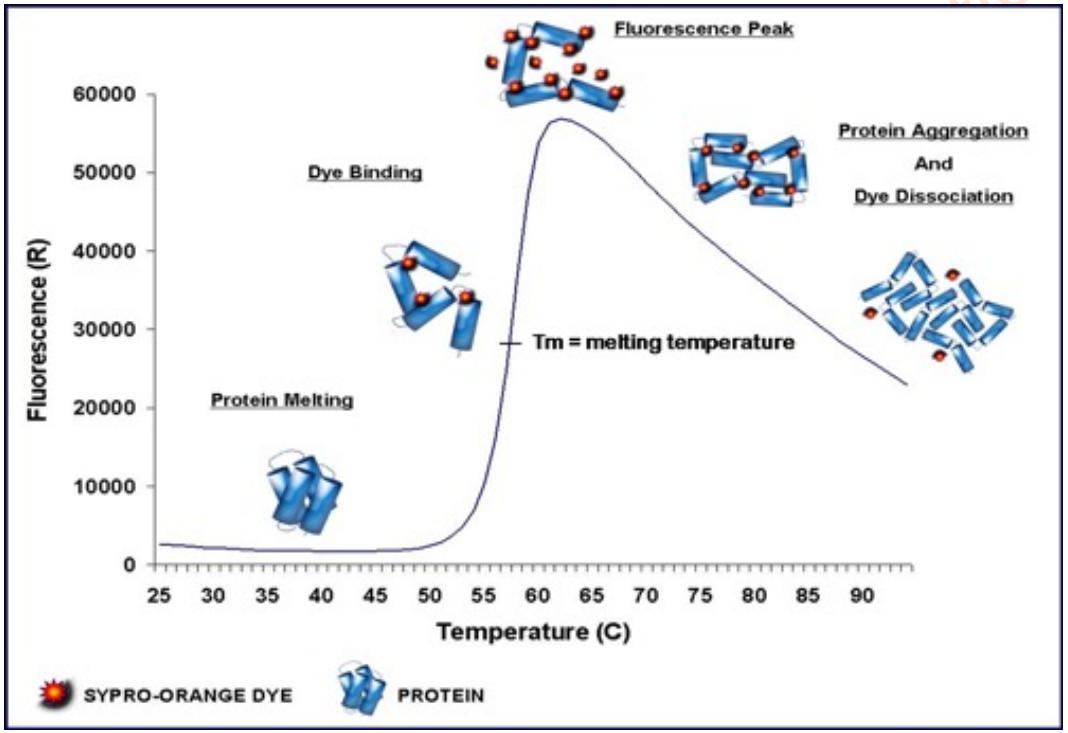
Electrospray ionisation - Mass spectrometry (ESI-MS)

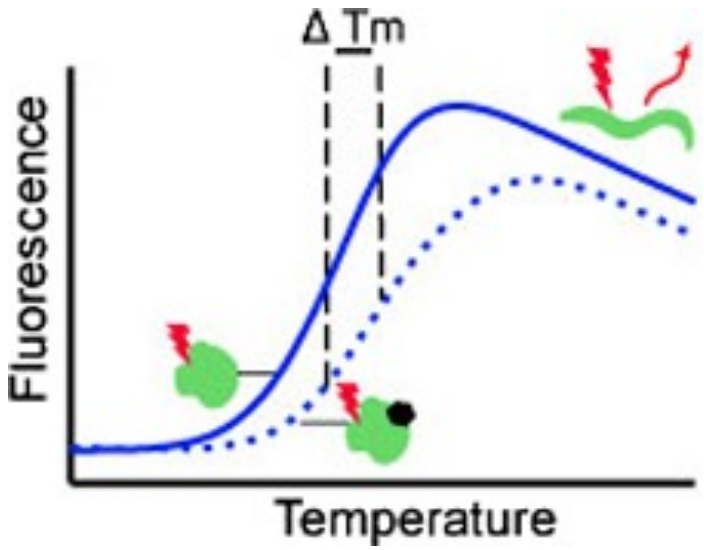
- Using ESI-MS, it is possible to transfer weakly associated complexes from solution into the gas phase inside the mass spectrometer source.
- ESI-MS not only provides a direct readout of binding stoichiometry but can also be used to determine dissociation constants ranging from nM to mM.
- The number of ligands bound for a given protein–ligand system can be determined directly from the spectrum based on the mass difference between free protein and its ligated complexes.
- In addition to exploiting the '*x* axis' of the mass spectrum (that is, the mass-tocharge ratio, *m/z*), the '*y* axis' of the mass spectrum (that is, abundance/intensity) provides important information about affinity and specificity.

- Pacholarz, Kamila J., et al. "Mass spectrometry based tools to investigate protein–ligand interactions for drug discovery." *Chemical Society Reviews*41.11 (2012): 4335-4355.
- Hofstadler, Steven A., and Kristin A. Sannes-Lowery. "Applications of ESI-MS in drug discovery: interrogation of noncovalent complexes." *Nature Reviews Drug Discovery* 5.7 (2006): 585-595.

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





Equilibrium dialysis

- The molecular weight cut off (MWCO) is chosen such that it will retain the receptor component.
- A known concentration and volume of ligand is placed into one of the chambers. The ligand is small enough to pass freely through the membrane.
- A known concentration of receptor is then placed in the remaining chamber in an equivalent volume to that placed in the first chamber.
- A complete binding curve is generated by measuring Y at different ligand concentrations.
- The relationship between binding and ligand concentration is then used to determine the number of binding sites, the ligand affinity, kd. Because this kind of experimental data used to be analyzed with (Scatchard plots)

Hatakeyama, Tomomitsu. "Equilibrium Dialysis Using Chromophoric Sugar Derivatives." *Lectins*. Springer New York, 2014. 165-171.

Affinity capillary electrophoresis (ACE)

- The technique uses the resolving power of CE to distinguish between free and bound forms of a receptor as a function of the concentration of free ligand.
- ACE experiments are most commonly performed in fused silica capillaries by injecting a receptor and neutral marker with increasing concentrations of ligand in the separation buffer.
- By studying the mobility change of a certain molecule when it interacts with another molecule of different mobility it is possible to determine the binding constant between the two compounds.
- The binding of the ligand to the receptor produces a migration time shift in the effective mobility due to a change in the charge:size ratio of the complex.
- Scatchard analysis of the effective mobilities measured as a function of ligand concentration provides the binding affinity of the receptor–ligand complex.

- Dinges, Meredith M., Kemal Solakyildirim, and Cynthia K. Larive. "Affinity capillary electrophoresis for the determination of binding affinities for low molecular weight heparins and antithrombin-III." *Electrophoresis* 35.10 (2014): 1469-1477.
- Chen, Zhi, and Stephen G. Weber. "Determination of binding constants by affinity capillary electrophoresis, electrospray ionization mass spectrometry and phase-distribution methods." TrAC Trends in Analytical Chemistry 27.9 (2008): 738-748.

Electrochemical methods

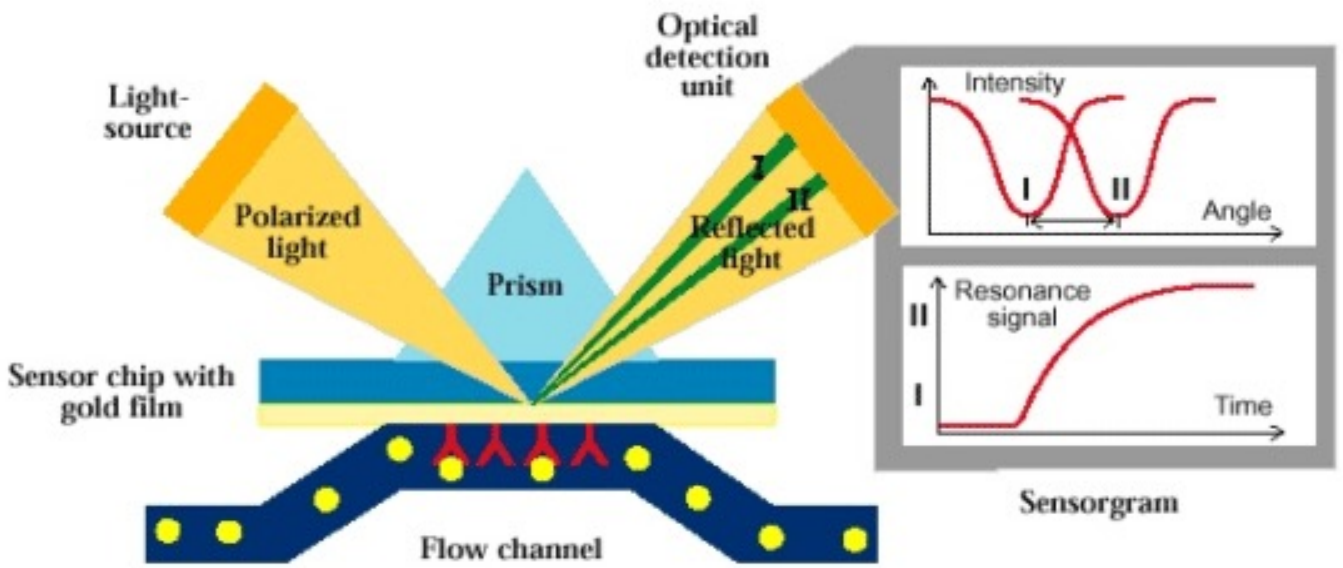
- Typically in (bio-)electrochemistry, the reaction under investigation:
  - Generate current (*amperometric*)
  - Generate potential or charge accumulation (*potentiometric*)
  - Alter the conductive properties of a medium (*conductometric*) between electrodes
  - Alter impedance

- NANO → The higher surface-to-volume ratio of nano-objects makes their electrical properties increasingly susceptible to external influences.

Grieshaber, Dorothee, et al. "Electrochemical biosensors-Sensor principles and architectures." *Sensors* 8.3 (2008): 1400-1458.

Surface plasmon resonance (SPR)

- Detection of molecular interaction on a chip surface
- Various set-ups: protein-protein, protein-ligand, protein-nucleic acid, protein-lipid membrane, protein-cell/virus



Surface plasmon resonance (SPR)

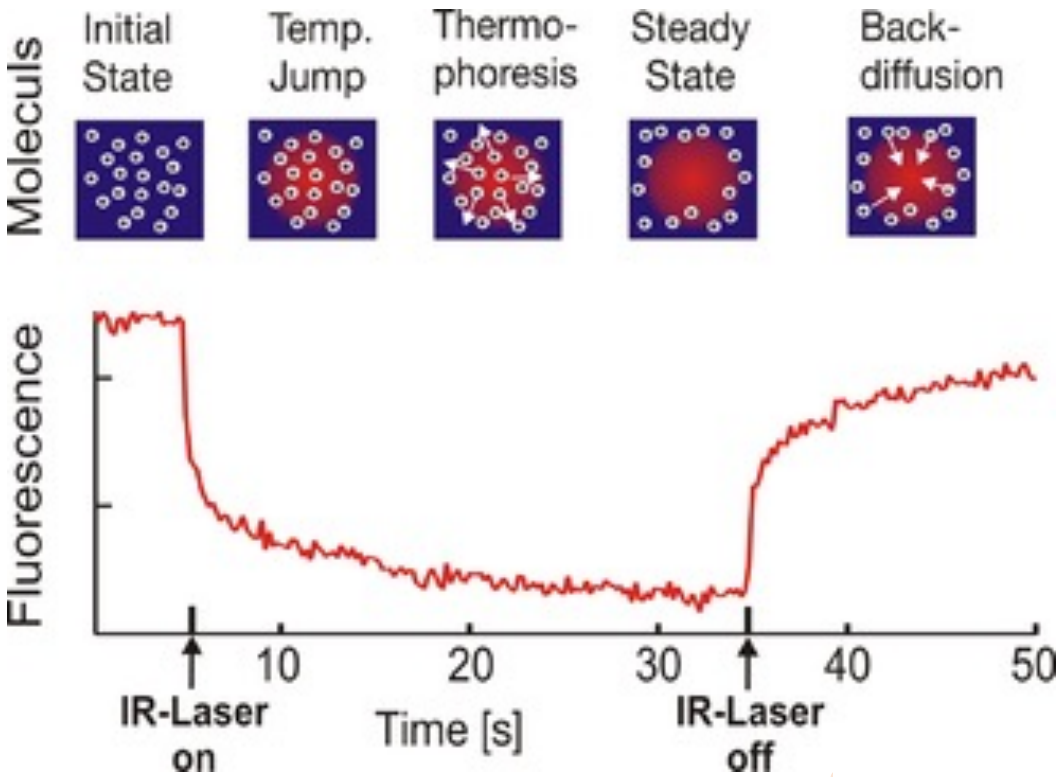
Advantage	Drawback
Label free	Tethering of molecules to surfaces may affect the binding constants measured
Enables quantitative determination	Any artifactual RI change other than from the interaction can also give signal
Low sample volume requirement	Stabilization process (in some cases)
Real time assay	
Sensitive	Cannot verify the stability of the complex formed

More tomorrow (Josef Houser)

Micro-scale thermophoresis (MST)

- It measures the motion of molecules along microscopic temperature gradients and detects changes in their hydration shell, charge or size.
- An infrared-laser is used to generate precise microscopic temperature gradients within thin glass capillaries that are filled with a sample in a buffer or bioliquid of choice.
- Thermophoresis, is very sensitive to changes in size, charge, and solvation shell of a molecule and thus suited for bioanalytics.
- The fluorescence of molecules is used to monitor the motion of molecules along these temperature gradients. The fluorescence can be either intrinsic (e.g. tryptophan) or of an attached dye or fluorescent protein (e.g. GFP).

Jerabek-Willemsen, Moran, et al. "Molecular interaction studies using microscale thermophoresis." *Assay and drug development technologies* 9.4 (2011): 342-353.



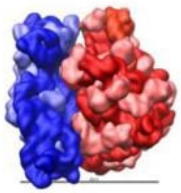
$c_{hot}/c_{cold} = \exp(-S_T \Delta T)$

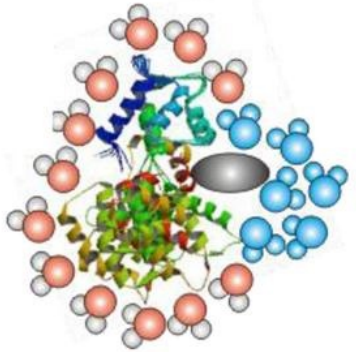
$$S_T = \frac{A}{kT}\left(-\Delta s_{hyd}(T) + \frac{\beta\sigma_{eff}^2}{4\pi\varepsilon\varepsilon_0 T} \times \kappa_{DH}\right)$$

size

charge ²

hydration shell

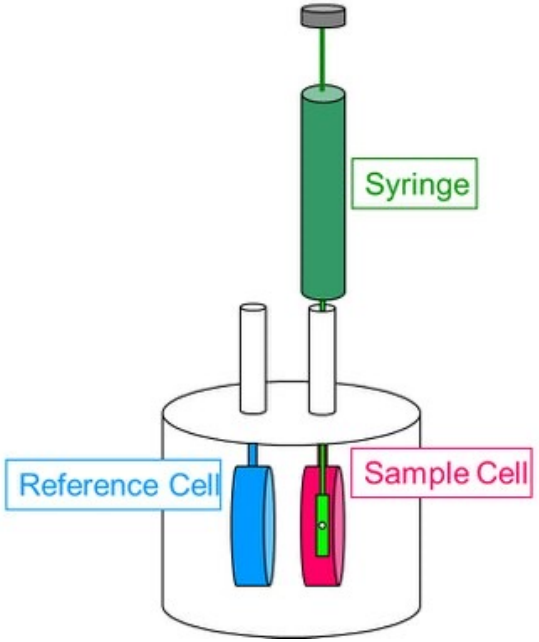




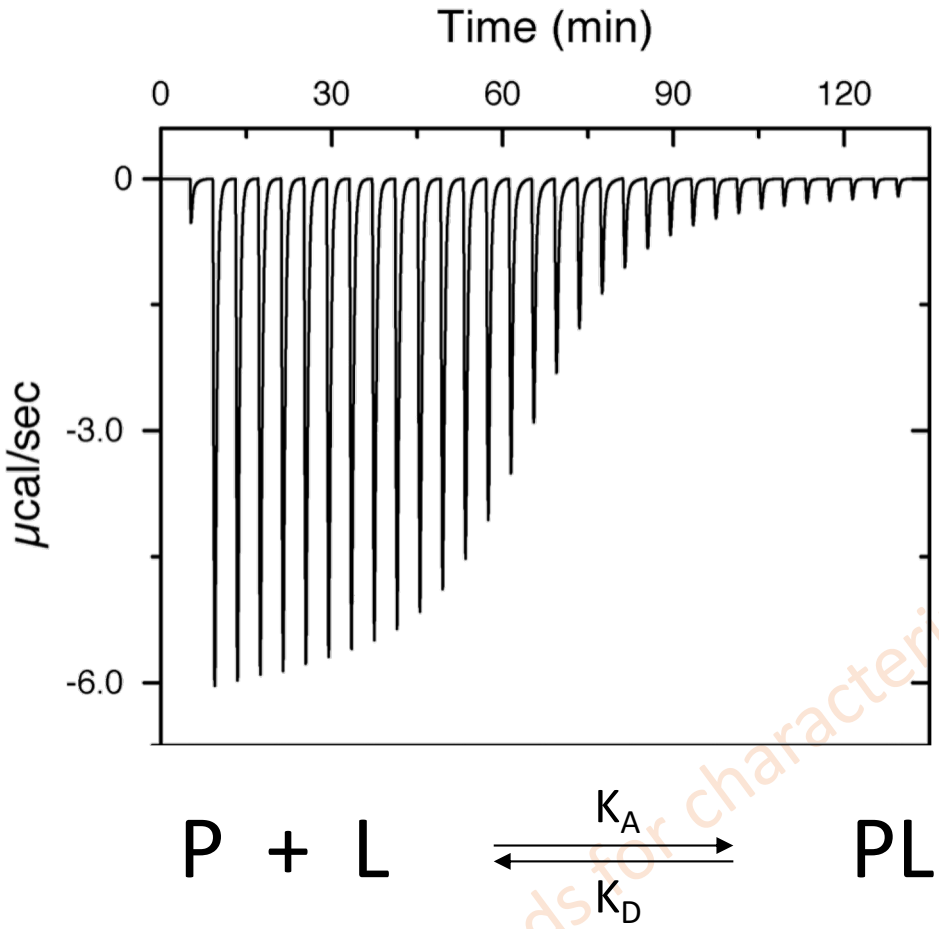
Advantage	Drawback
Sample concentration (pM/nM) and small volume (< 4 µl)	Buffer condition must be absolutely stable
Quantitative (K: pM/nM to mM range and n)	Conformational changes induced by IR-Laser heating may be problematic
measures interactions with essentially no limitation on molecule size or molecular weight.	-
Immobilization free	-
Free in choosing buffer type	-

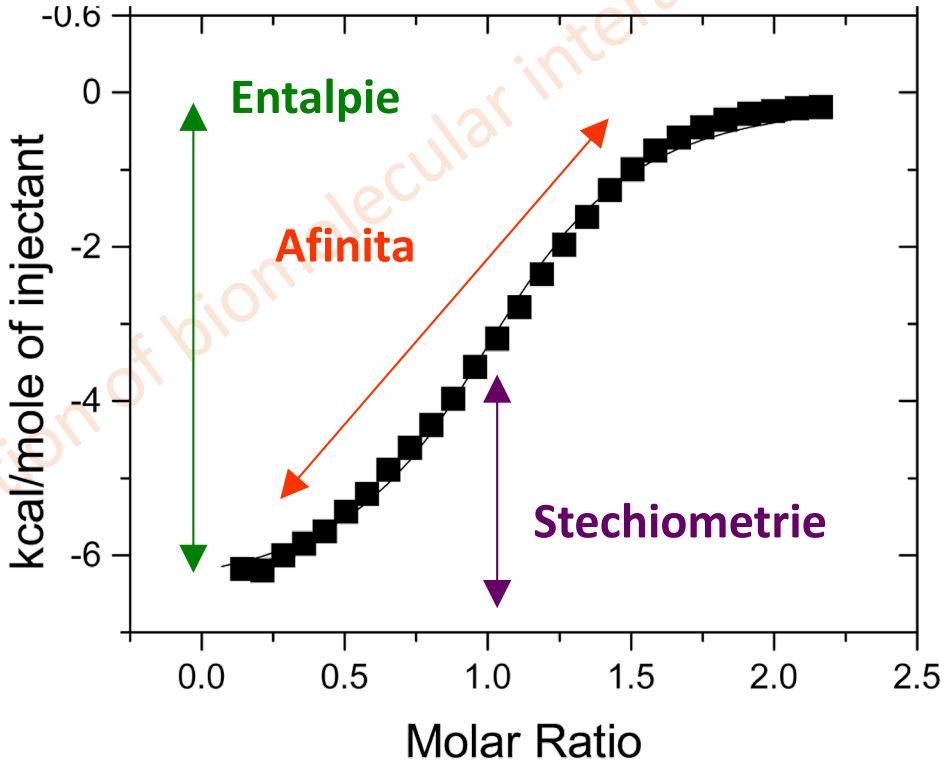
More later today (Eva Fujdiarová)

Isothermal titration calorimetry (ITC)



- All chemical, physical and biologic processes are performed along with heat exchange criteria.
- When a protein interacts with a ligand, heat is either released or absorbed.
- ITC relies only on the detection of a heat effect upon binding → not relies on the presence of chromophores or fluorophores.
- Can be used to measure the binding constant, the enthalpy of binding, and the stoichiometry.





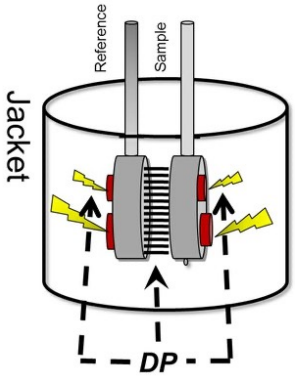
$$\Delta G^\circ = -RT \ln K_A$$

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

Advantage	Drawback
Label free	Large sample volumes required
Enables quantitative determination (K and n)	High ligand concentrations
Can be done on solutions that are either homogeneous or heterogenous	Presence of impurities or inactive protein will have a direct impact on the stoichiometry
Universal	-

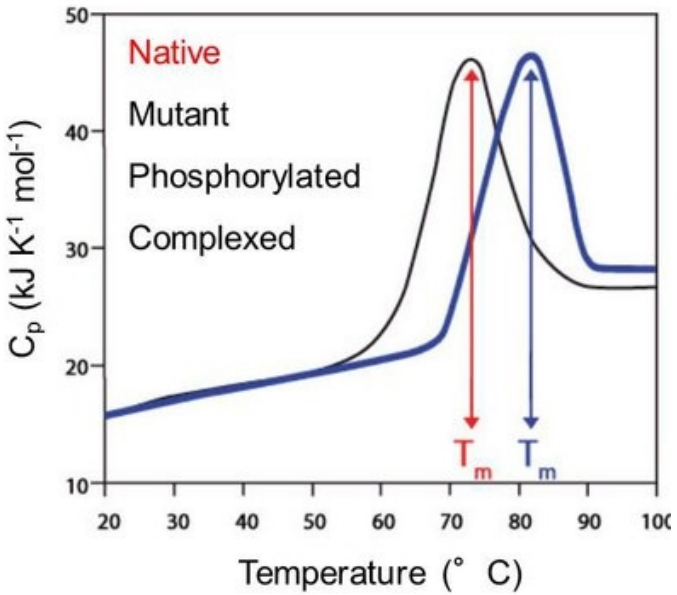
More later today – (Monika Kubíčková)

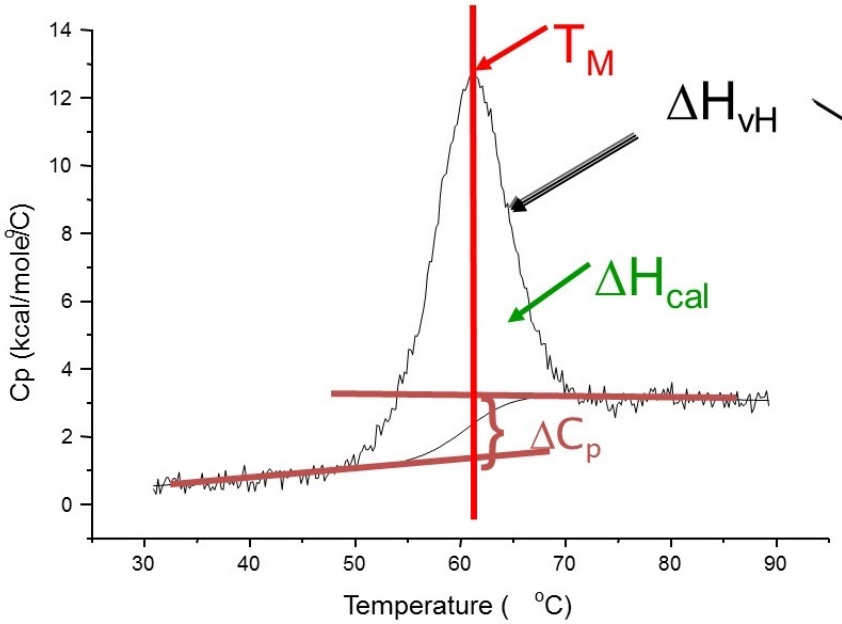
Differential scanning calorimetry (DSC)

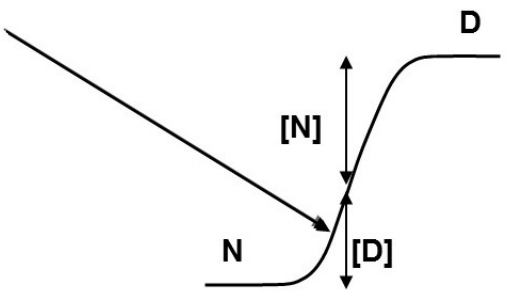


- Measures heat capacity in a range of temperatures.
- If a ligand binds preferentially to the native state of the protein, the temperature at which the protein-ligand complex denatures will be high compared to the temperature at which the free protein unfolds.
- Since the degree of stabilization or destabilization of the native protein depends on the magnitude of the binding energy, comparison of the stability of the complex with the stability of the ligand-free protein allows the binding energy to be estimated.
- DSC thus provides a direct measure of whether ligand binding to a protein is stabilizing or destabilizing, and so can complement studies of binding equilibria obtained by isothermal titration calorimetry (ITC).

Chiu, Michael H., and Elmar J. Prenner. "Differential scanning calorimetry: an invaluable tool for a detailed thermodynamic characterization of macromolecules and their interactions." *Journal of Pharmacy And Bioallied Sciences* 3.1 (2011): 39.







Advantage	Drawback
Label free	Sensitivity depends on many parameters
Quantitative (relatively)	useful in characterizing very tight binding interactions which equilibrate very slowly (mins to hrs)
Gives information on the nature of binding event	-

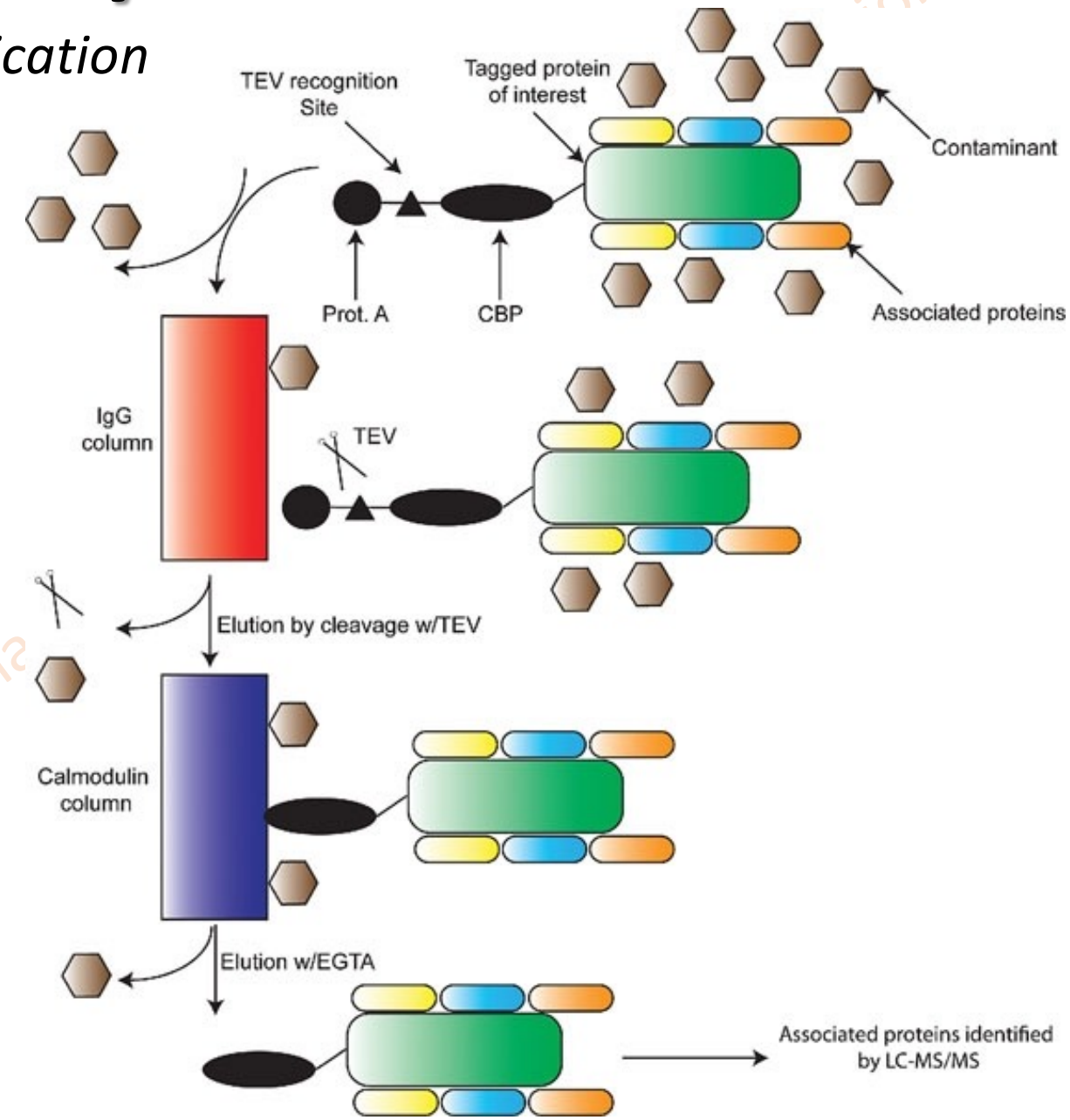
Complex techniques

- Indirect detection of molecular interaction
- Multi-step approaches

Pull-down assay

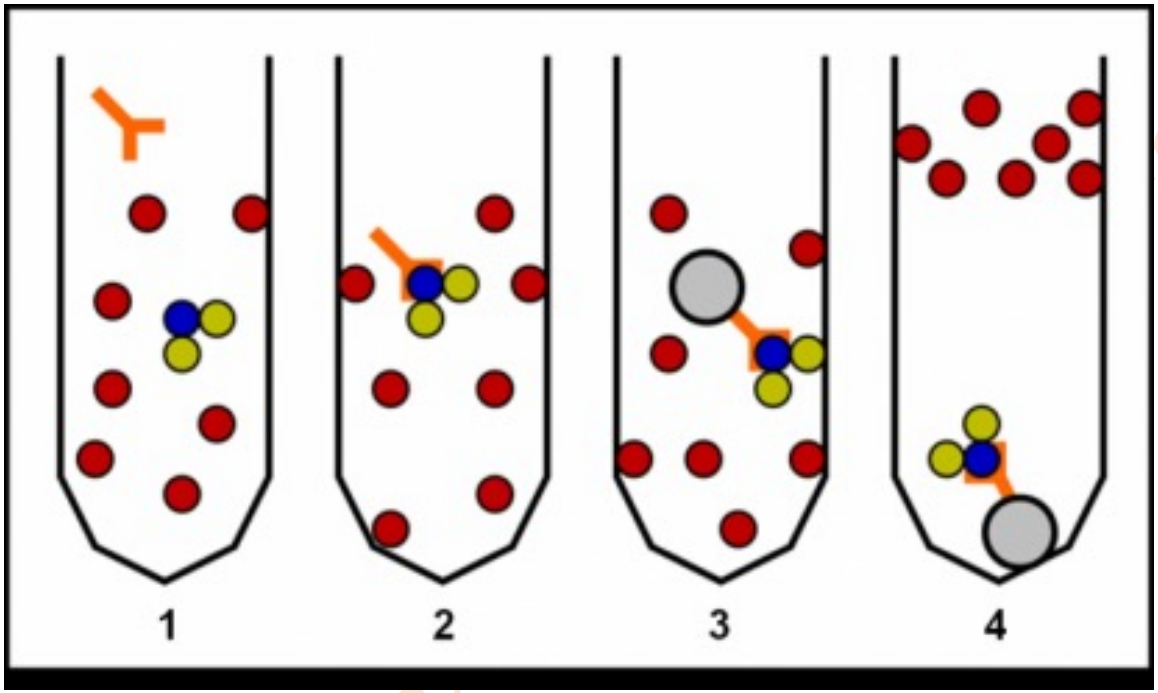
Tandem affinity purification

Identification of protein-protein interaction



Puig O et al (2001) Methods. Jul;24(3):218-29

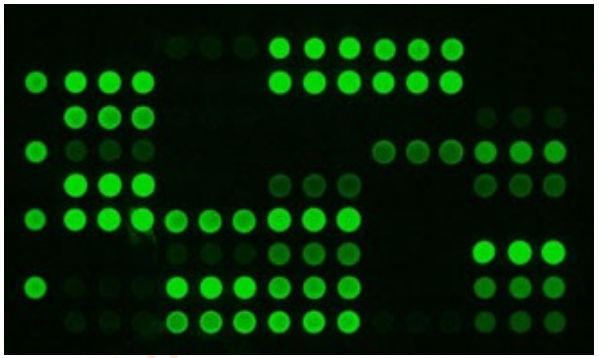
Co-immunoprecipitation



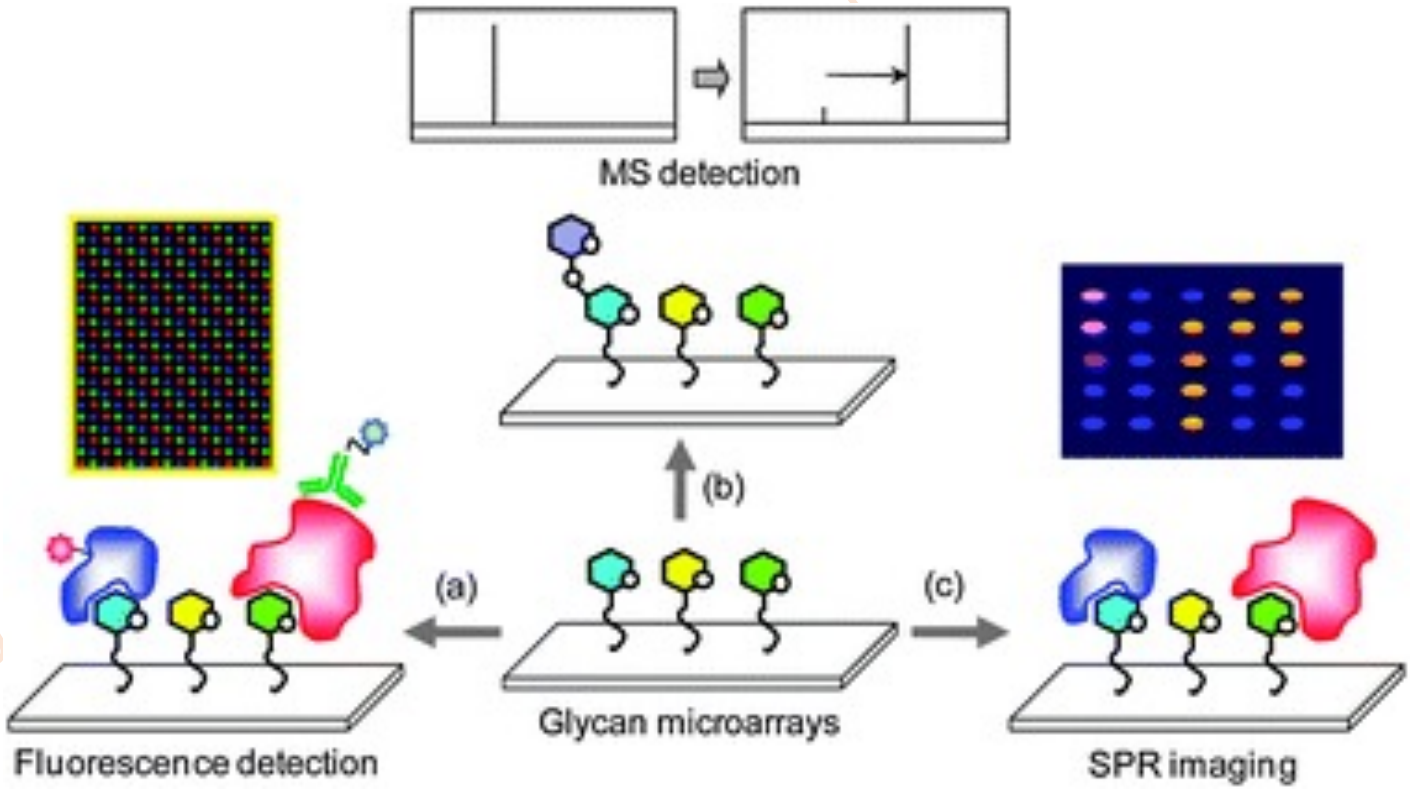
MS analysis

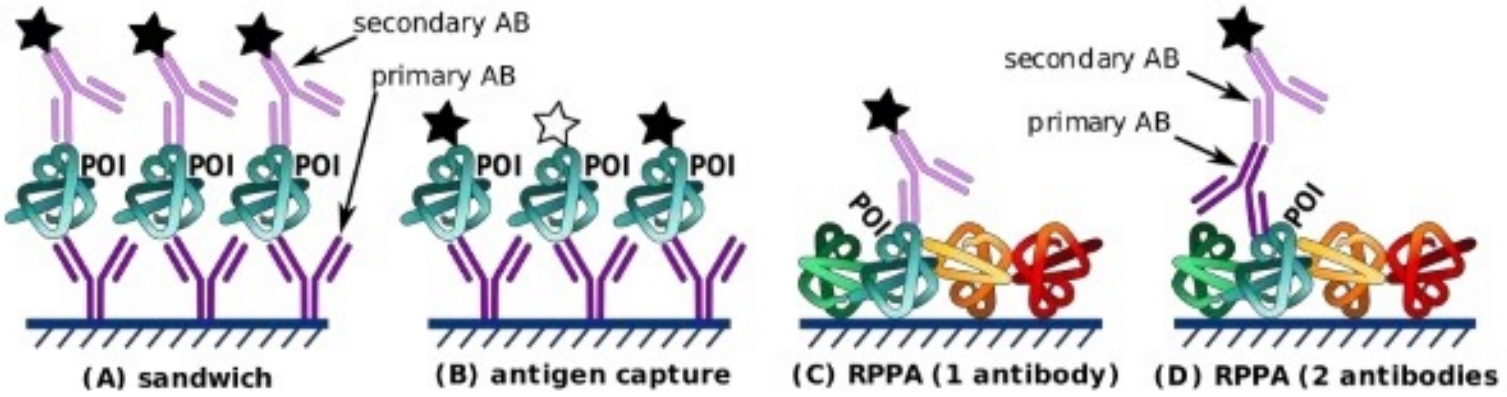
[1] Addition of antibody to protein extract. [2] Target proteins are immunoprecipitated with the antibody. [3] Coupling of antibody to beads. [4] Isolation of protein complexes.

Microarrays

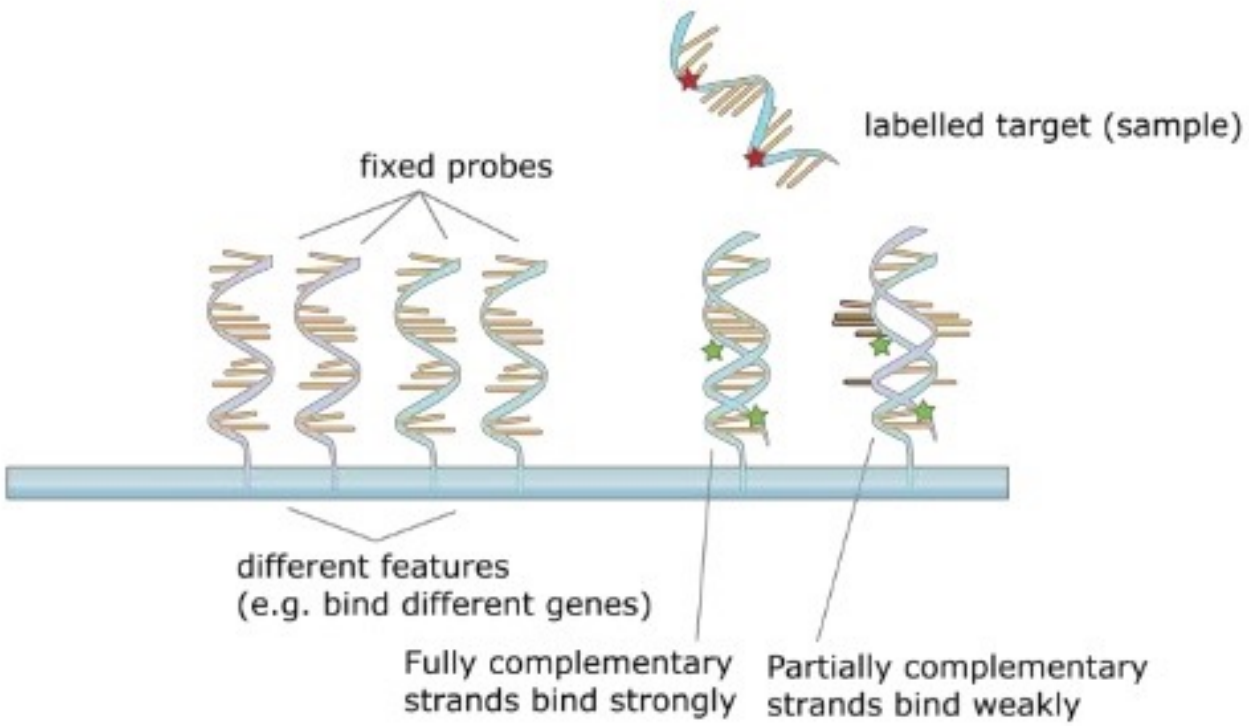


- High screening capacity possible
- Semi-quantitative



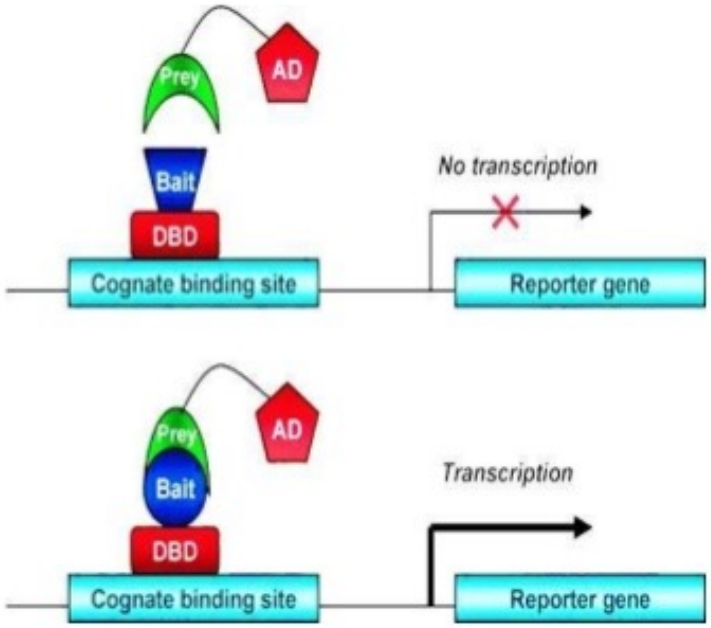


- Various immobilized molecules
  (protein, nucleic acid, saccharide)



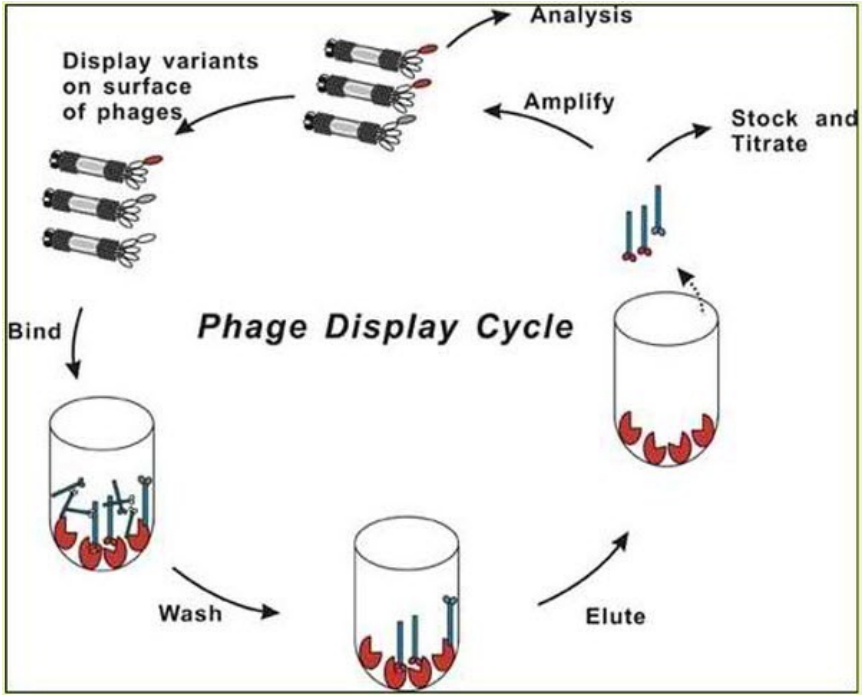
Yeast two-hybrid system

- Testing for physical interactions between two proteins or protein/DNA.
- Is based on the properties of the yeast GAL4 protein, which consists of separable domains responsible for DNA-binding and transcriptional activation.
- Plasmids encoding two hybrid proteins, one consisting of the GAL4 DNA-binding domain fused to protein X and the other consisting of the GAL4 activation domain fused to protein Y, are constructed and introduced into yeast.
- Interaction between proteins X and Y leads to the transcriptional activation of a reporter gene containing a binding site for GAL4.



- **AD**: activation Domain
- **DBD**: DNA Binding Domain
- **Reporter gene**:
  LacZ reporter - Blue/White Screening

Phage display

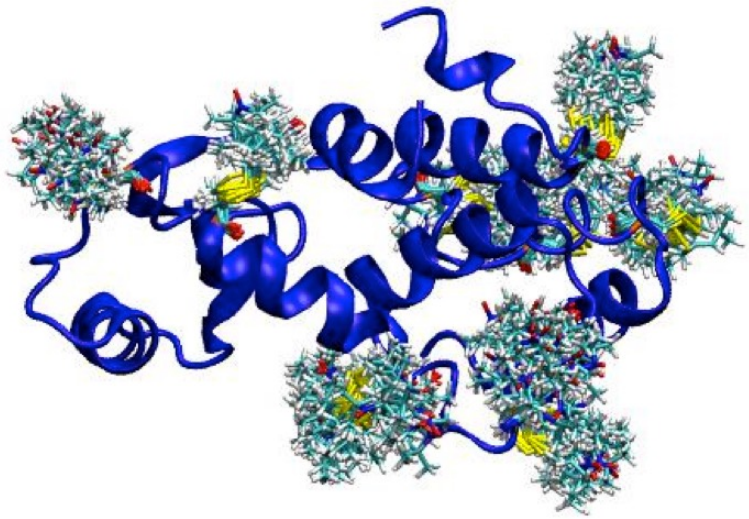


- For the study of protein–protein, protein–peptide, and protein DNA interactions.
- A gene encoding a protein of interest is inserted into a phage coat protein gene, causing the phage to "display".
- These displaying phages can then be screened against other proteins, peptides or DNA sequences to detect interaction.
- The most common bacteriophages used in phage display are M13 and filamentous phage, though T4, T7, and λ phage have also been used.

Bratkovič, Tomaž. "Progress in phage display: evolution of the technique and its applications." *Cellular and molecular life sciences* 67.5 (2010): 749-767.

Computational methods

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



➢ Relatively cheap

➢ Less accurate

➢ Ideally to be combined with experimental approaches

Take home message

- Many techniques available
- Various principles, sample requirements, detection limits,…
- There is no single ideal method
- Method knowledge is crucial to get the best results

Methods for characterization of biomolecular interactions – classical versus modern (autumn 2021)

Teachers: Josef Houser, Monika Kubíčková, Jan Komárek, Eva Fujdiarová, Michaela Wimmerová

Students enrolled in S2004 course attend only lectures (L). Students enrolled in both S2004 and S2005 courses need to attend both lectures (L) and practical exercises (P).

February 1, 2022 (Tuesday)

time		program	room
9:00-10:30	L	Biomolecular interactions – introduction *(Wimmerová)*	A5/114
10:30-10:45		break	
10:45-12:15	L	Isothermal titration calorimetry (ITC) *(Kubíčková)*	A5/114
12:15-13:15		lunch	
13:15-14:00	L	Spectroscopic methods *(Houser)*	
14:00-15:00	L	Microscale thermophoresis (MST) *(Fujdiarová)*	A5/114
15:00-15:30		break	
15:30-17:00	P	ITC – hands-on *(Kubíčková)*	A4/218

February 2, 2022 (Wednesday)

time		program	room
9:00-10:30	L	Analytical ultracentrifugation (AUC) *(Komárek)*	A5/114
10:30-10:45		break	
10:45-12:15	L	Surface-based methods *(Houser)*	A5/114
12:15-13:15		lunch	
13:15-13:45	L	Interactions at the cell level *(Houser)*	A5/114
13:45-14:45	L	Importance of sample preparation *(Houser)*	A5/114
14:45-15:00		break	
15:00-15:30	P	ITC – data analysis *(Kubíčková)*	A4/218
15:30-17:30	P	AUC – hands-on *(Komárek)*	A4/217

February 3, 2022 (Thursday)

time		program	room
9:00-10:00	P	AUC – data analysis *(Komárek)*	A4/217
10:00-10:15		break	
10.15-11.30	P	MST – hands-on *(Fujdiarová)*	A4/219
11:30-12:30		lunch	
12:30-13:00	P	MST – data analysis *(Fujdiarová)*	A4/219
13:00-14:30	P	BLI – hands-on *(Houser)*	A4/218
10:30-10:45		break	
15:00-16:00	P	BLI – data analysis *(Houser)*	A4/218
16:15-17:00	P	**Discussion of practical aspects** *(all teachers)*	A4/218

February 4, 2022 (Friday)

time	program	room
9:30-11:00	**Written test**	A4/211