Central European Institute of Technology
BRNO | CZECH REPUBLIC

Biomolecular interactions

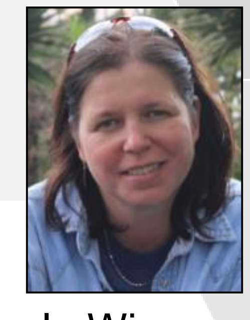
Josef Houser

Glycobiochemistry & Biomolecular Interaction and Crystallization CF
CEITEC

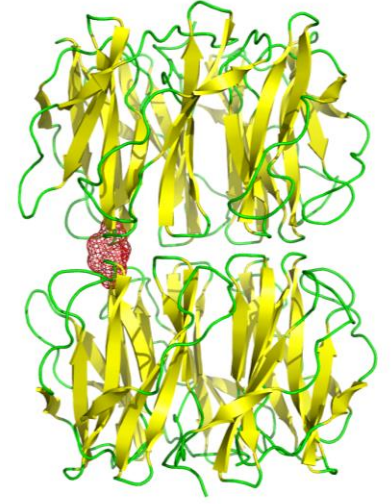
Who am I?

Josef Houser

- Glycobiochemistry
- Biomolecular Interaction and Crystallization CF



Prof. Michaela Wimmerová



Biomolecular interactions

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.

Biomacromolecules

Biomolecules

Macromolecules

Saccharides
Hormones
Pigments
Vitamines
Alcaloides

Proteins
Nucleic acids
Polysaccharides

Plastics
Graphene
Nanotubes

Composition of biomacromolecules

They are formed by linking a huge number of subunits of several types into one chain

Macromolecules	Building blocks	Type of bond	Scheme
Protein	Amino acids	Peptidic	O R O NH NH NH O R
Nucleic acid	Nucleotides	Ester	B O O O P O OH B O O O P O OH
Polysaccharide	Monosaccharides	Glycosidic	OH O HO OH O OH O HO OH O OH O HO OH

Amino acids

Acidic Basic Polar

Aromatic Hydrophobic

Valine, Tryptophan, Asparagine, Lysine

Glycine, Phenylalanine, Methionine, Aspartic Acid, Leucine, Serine, Glutamine, Arginine

Alanine, Tyrosine, Cysteine, Glutamic Acid, Isoleucine, Threonine, Proline, Histidine

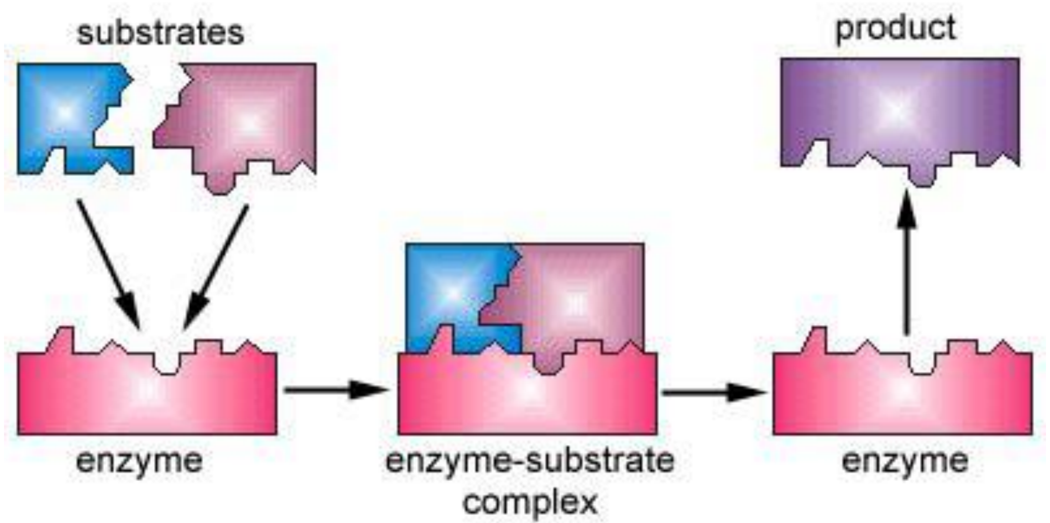
glycine	alanine	valine	leucine	isoleucine	aspartic acid	asparagine	glutamic acid	glutamine	arginine	lysine	histidine	phenylalanine	serine	threonine	tyrosine	tryptofan	methionine	cysteine	proline	selenocysteine	pyrolysine
Gly	Ala	Val	Leu	Ile	Asp	Asn	Glu	Gln	Arg	Lys	His	Phe	Ser	Thr	Tyr	Trp	Met	Cys	Pro	Sec	Pyr
G	A	V	L	I	D	N	E	Q	R	K	H	F	S	T	Y	W	M	C	P	U	O

Biomolecular interactions are everywhere…

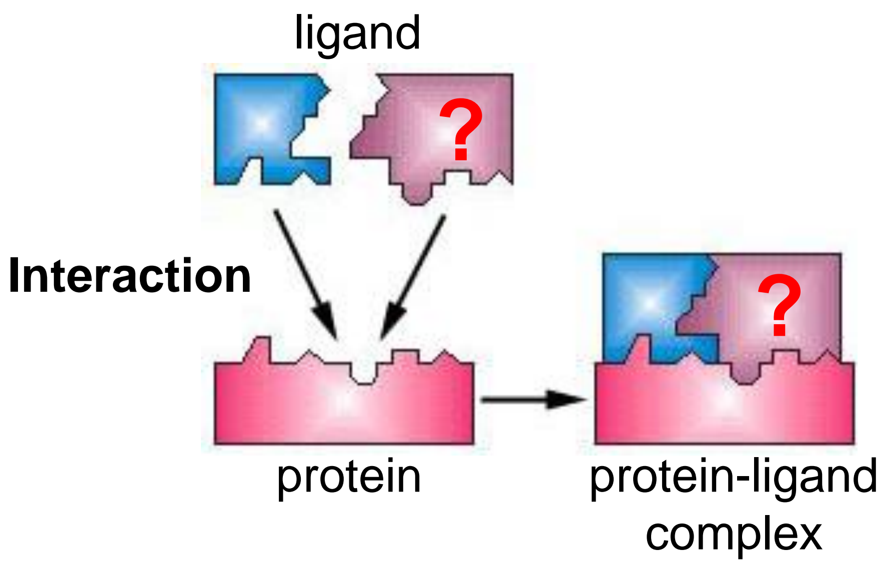
Protein – Ligand	Protein – Solvent
Protein – Protein	Nucleic acid – Solvent
Protein – Nucleic acid	
Nucleic acid – Ligand	Protein – Inorganic salt
Protein/NA adsorption	Nucleic acid – Inorganic salt

All processes in living organisms are essentially determined by biomolecular interactions

Interaction vs. chemical reaction



Interaction vs. chemical reaction



Types of interaction

- **Nuclear physics**

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

- **Chemistry** (electron ionization)

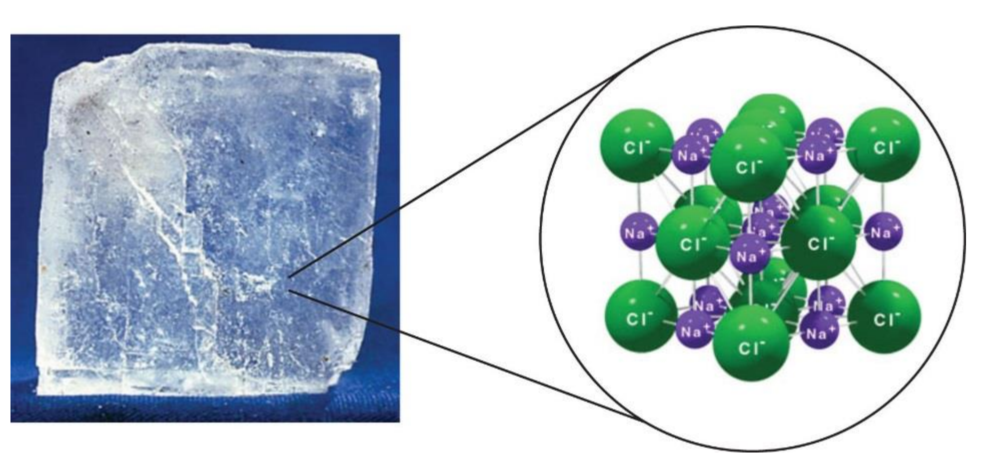
  formation of bonds 150-1000 kJ/mol

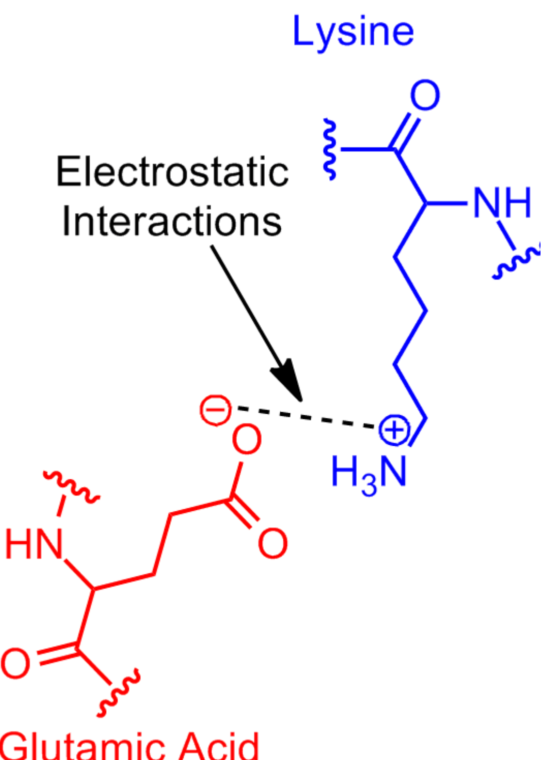
- **Biochemistry-biology**

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

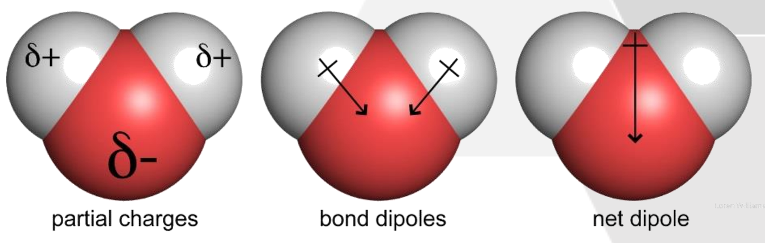
Coulombic interactions (salt bridge)

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





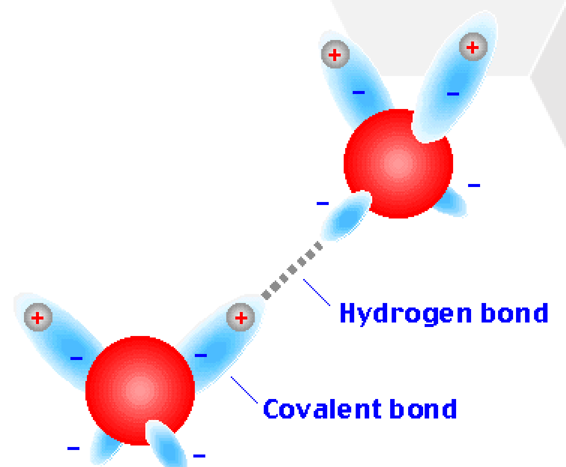
Dipole interactions



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

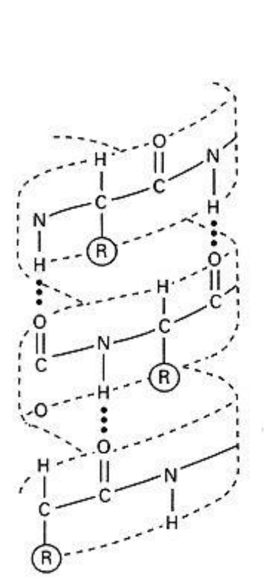
Hydrogen bonds

- Atom with free electron pair + hydrogen bound to electronegative atom (O, N, X, S, C, ...)



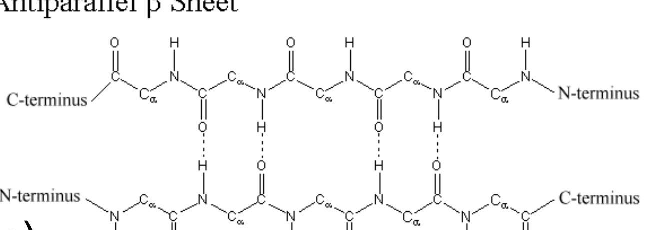
G C

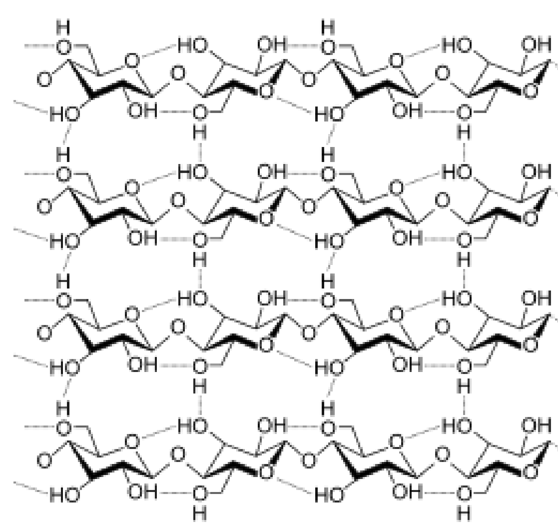
DNA (base pairing)



Protein
(2D structure stabilization)

Antiparallel β Sheet



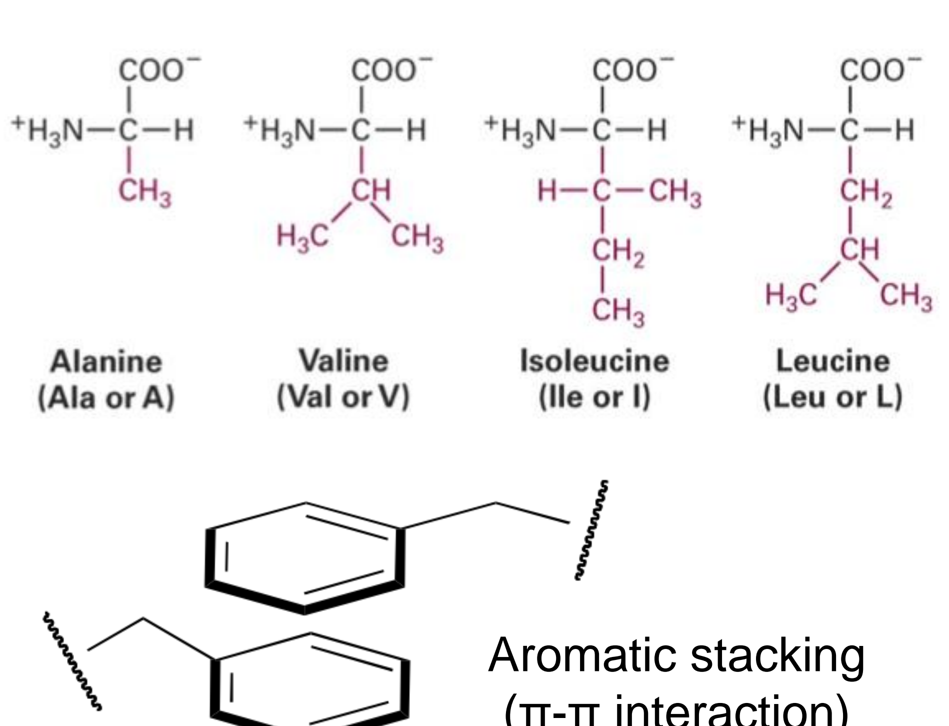


Polysaccharide (cellulose)

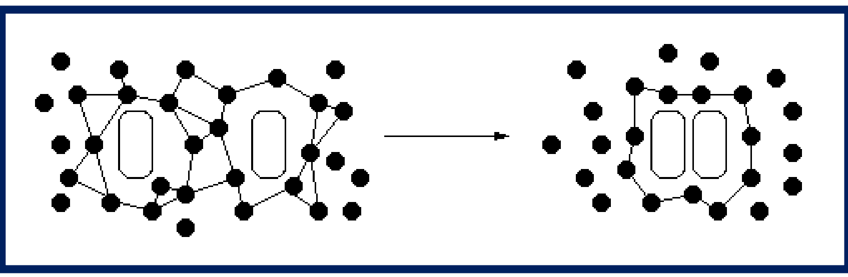
Hydrophobic interactions

van der Waals, nonpolar interactions

- Driven by entropy – strong influence of temperature



Aromatic stacking
(π-π interaction)



Mostly more than one effect is present

H-bonds

O

^{-}N

N^{+}

CH_3

$C_{10}H_{23}$

Hydrophobic/
Steric

Charge/
Acid-Base

Charge

Hydrophobic

Interaction description

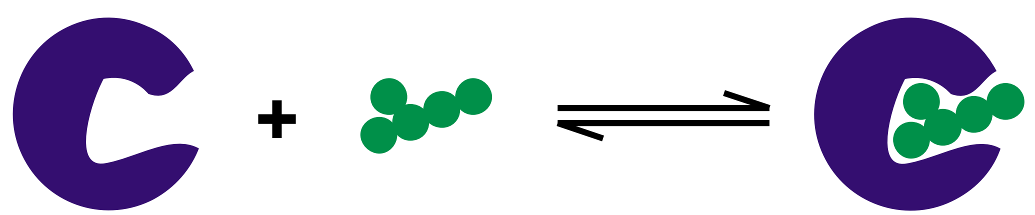
Physics

Mathematics

Physical chemistry

Biology

Receptor – ligand interaction

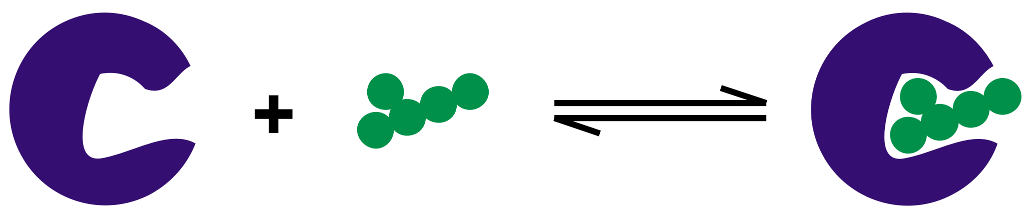


$$\frac{d[\mathrm{MX}]}{dt} = k_a[\mathrm{M}][\mathrm{X}] - k_d[\mathrm{MX}]$$

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

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

Gibbs energy, enthalpy, entropy



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

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

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

$\Delta G < 0$	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

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

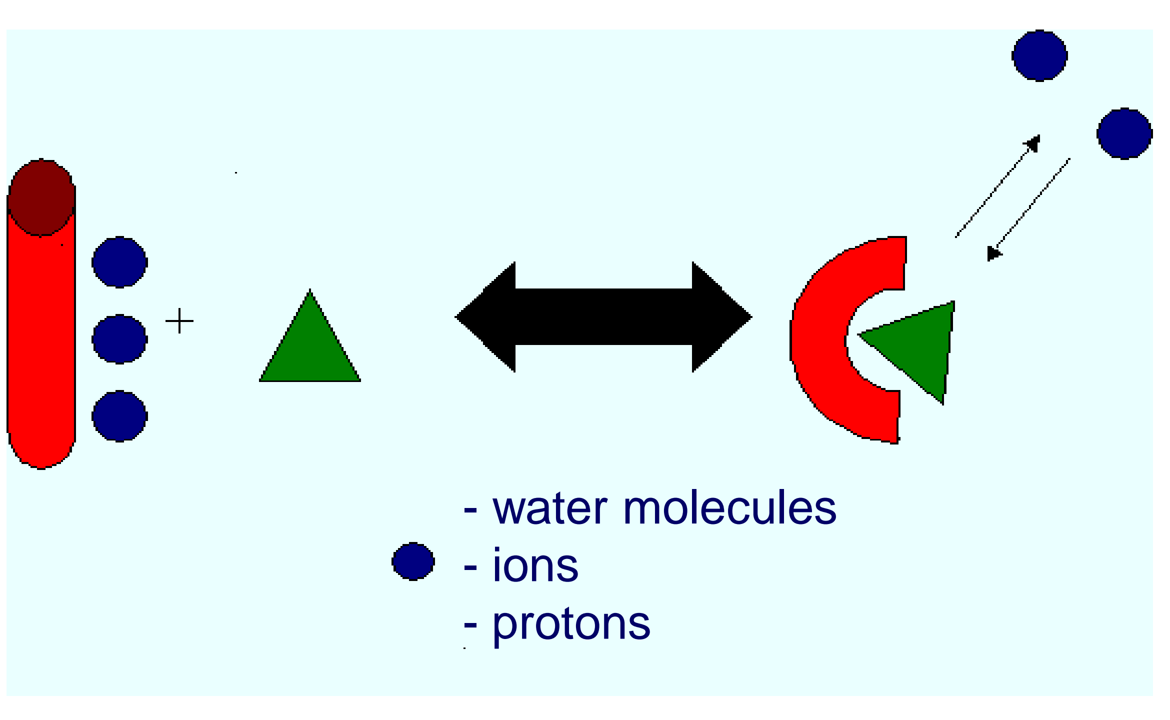
Why to study the interactions?

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

- 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

Rational drug design – Energetic contributions involved



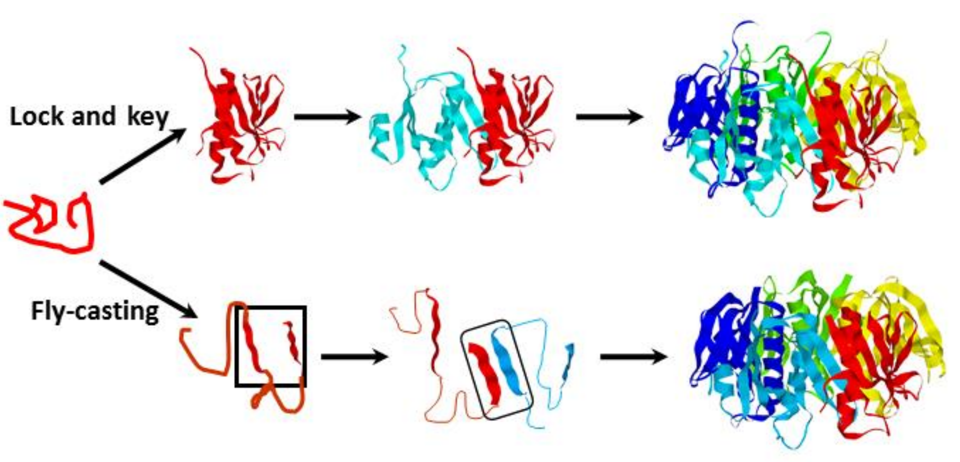
Enthalpy
- Hydrogen bonds
- Protonation

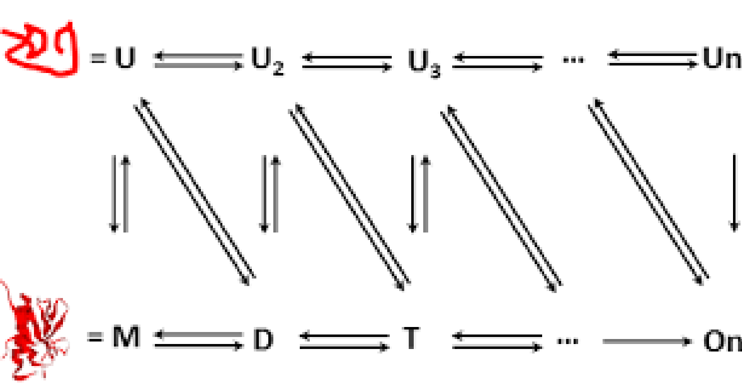
Entropy
- Hydrophobic interactions
- Water release
- Ion release
- Confromational changes

Oligomerization

- Special type of interaction with identical molecule

$$A + A \rightarrow A_2 \qquad K_D = \frac{k_d}{k_a} = \frac{[A][A]}{[A_2]} = \frac{[A]^2}{[A_2]}$$





Lesieur C 2013 Oligomerization of Chemical and Biological Compounds

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

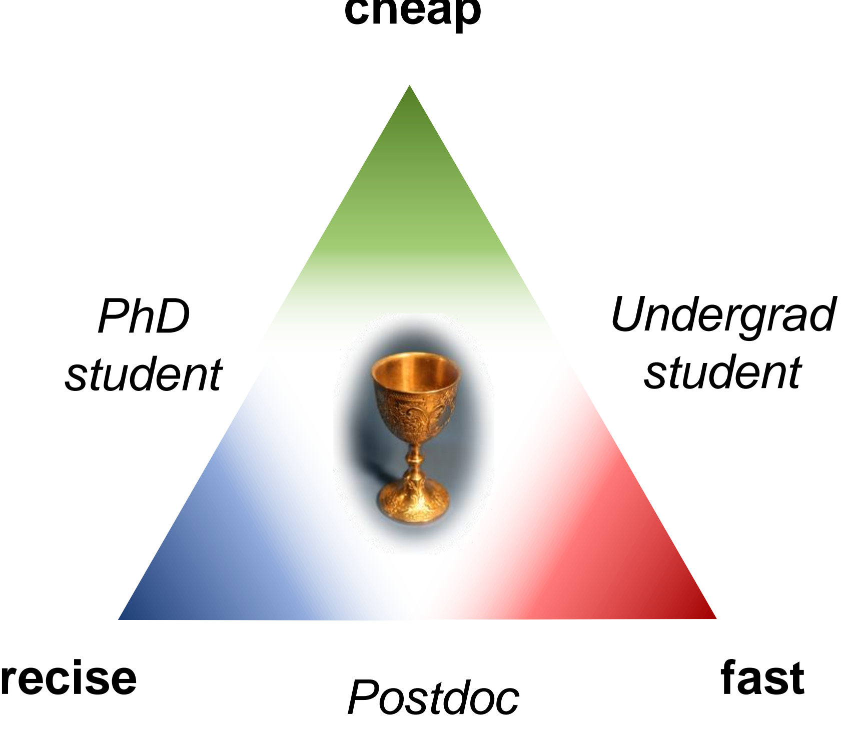
Experimental methods to study biomolecular interactions

Experimental techniques to measure the interactions

- Physical background

- Information content
- Speed of analysis
- Suitable system studied

- Availability
- Complementarity
- "Fashion"



Physical properties in background

Process that **AFFECTS** the molecules (to reveal difference of free and bound) need not to be the same used for **OBSERVING** the molecules

Nuclear spin (NMR)

Electrical properties

Fluorescence

Refractive index (SPR)

Heat (ITC)

Sedimentation (AUC)

Spatial distribution (dialysis)

Mobility (chromatography, electrophoresis)

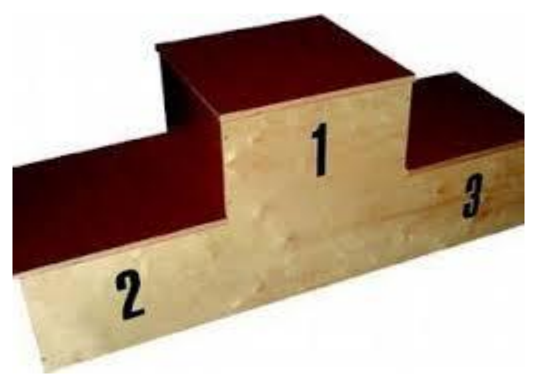
Electron spin (EPR)

Mass (MS)

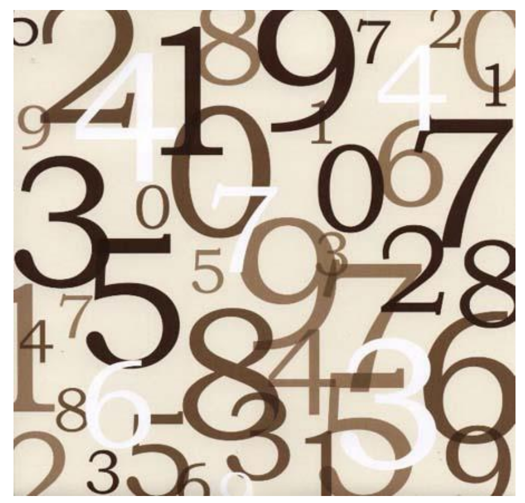
Two informational levels of methods

Qualitative

Semi-quantitative



Quantitative



Suitability for particular system

Specific labeling

- GFP co-expression
- covalent attachment (amino coupling)
- non-covalent (His-tag)

Immobilization

- covalent (amino coupling)
- capture (Ab, His-tag, streptavidine-biotin)

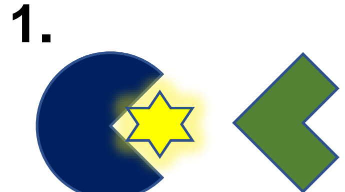
Buffer compatibility

- ionic strength
- interfering components
- pH

Which binding partner to label?

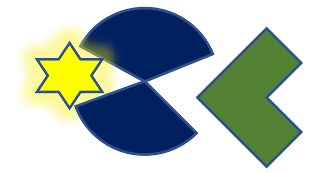
Interference with interaction

1.



1. Sterical hindrance

2.

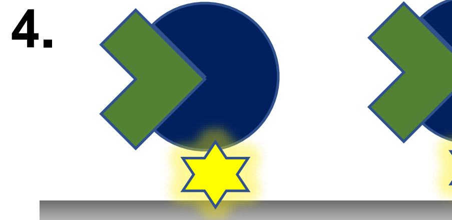


2. Conformation changes

3.

3. Non-specific interaction

4.

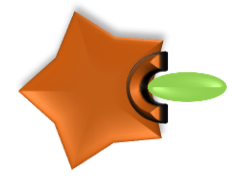
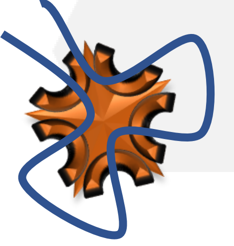


4. Adhesion to labware

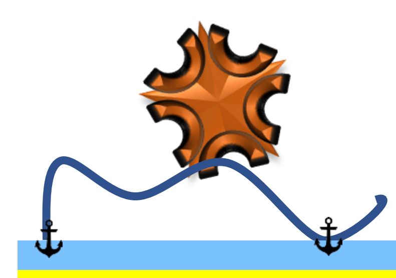
5.

5. Solubility change, aggregation

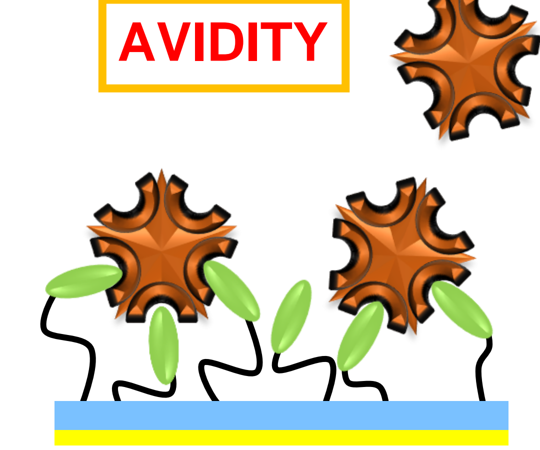
Immobilization

- **Sterical hindrance**
  - Binding site not accessible
  - Restricted movement
  - **Distorted conformation**



- **Multivalency**
  - Non-equivalent accessibility of binding sites
  - Avidity vs Affinity



Availability (Where? How much?)

CEITEC Core Facilities

- **Biomolecular Interaction and Crystallization**
- **Josef Dadok National NMR Centre**
- **Nanobiotechnology**
- Proteomics
- Cellular Imaging
- Cryo-Electron Microscopy and Tomography

Financial support

Instruct, CIISB, ...

Complementarity

No method is perfect

Endothiapepsin binding to small-molecule library

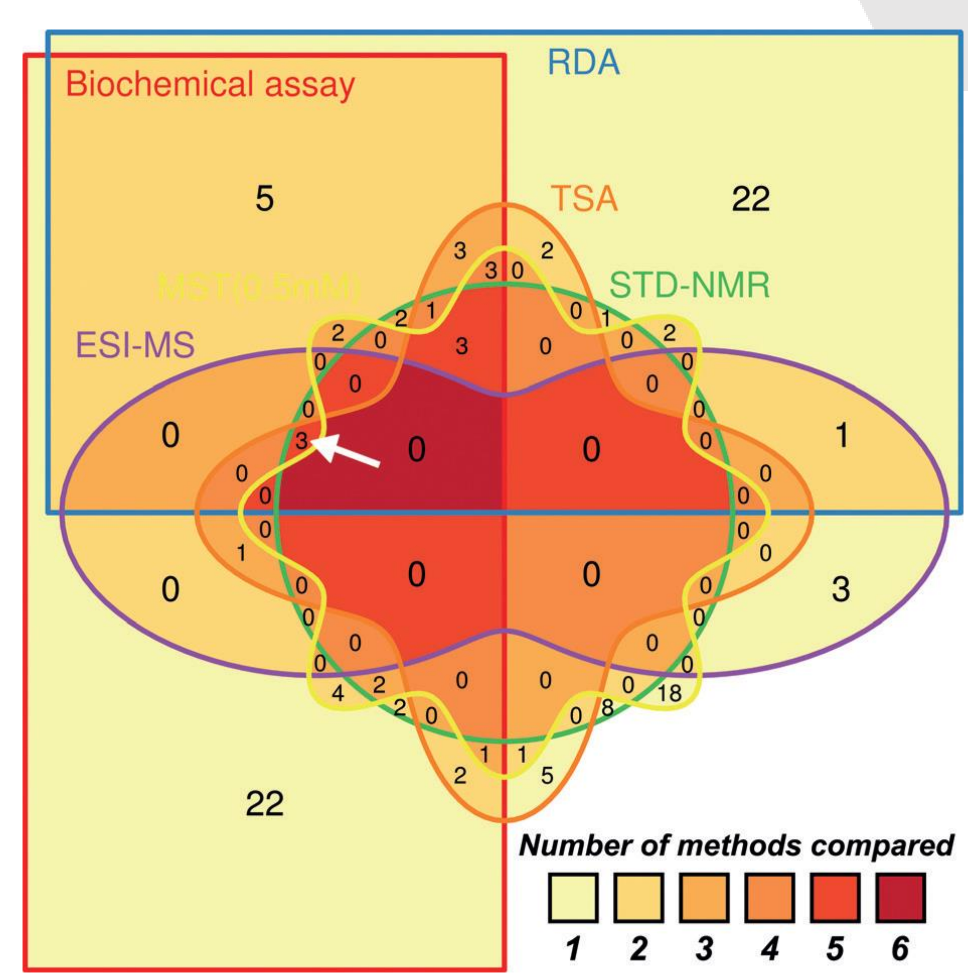
361 compounds tested

239 potential binders (≥1 method)

161 identified by ≥2 methods

6 identified by 5 methods

0 identified by all 6 methods !!!

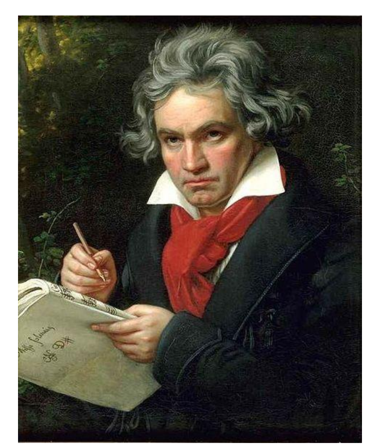


Schiebel J *et al* **2015**, *ChemMedChem*

Fashion (What is IN?)

Classical vs. Modern

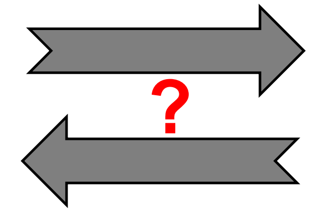
What is "classical"?



What is "modern"?

Fashion (What is IN?)

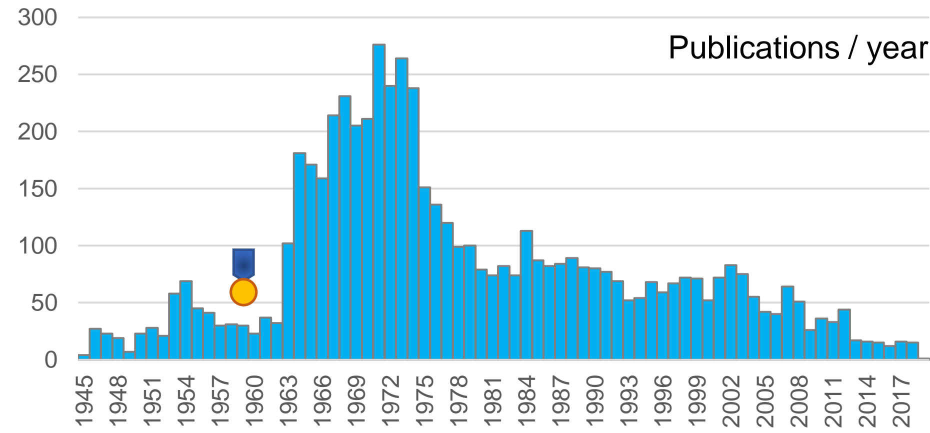
Famous method



Nobel prize award

Polarography

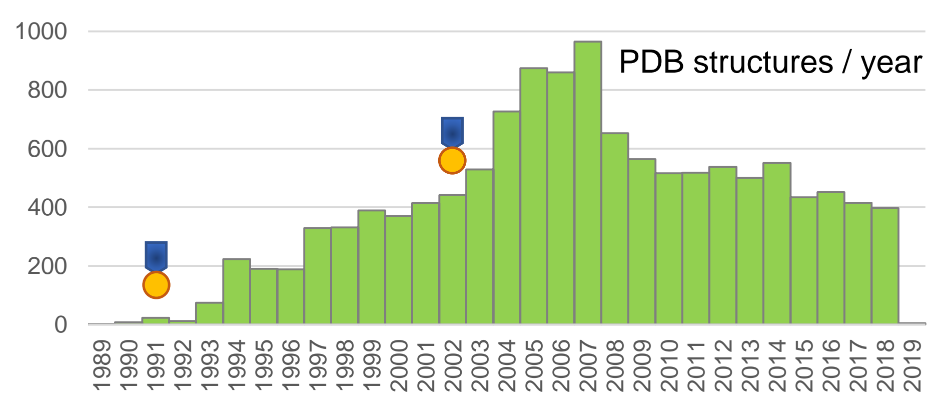
1959 Jaroslav Heyrovský



NMR

1991 Richard R. Ernst

2002 Kurt Wüthrich



Experimental techniques to measure the interactions

Examples

16:00 Lab tour: Biomolecular Interactions and Crystallization core facility

THURSDAY January 10

9:00 Lecture 9 Markus Hartl: A primer on structural proteomics: MS-basics, cross-linking, HDX, ion-mobility

10:30 Tea & coffee (A11/205)

11:00 Lab tour: Cryo-Electron Microscopy and Tomography and Nanobiotechnology core facilities

12:30 Lunch (A35 Atrium)

14:00 Lecture 11 Edward Lemke: Tools to decode molecular plasticity in the dark proteome

15:30 Tea & coffee (A11/205)

16:00 Lab tour: Proteomics and Cellular Imaging core facilities

FRIDAY January 11

9:00 Lecture 12 Sonia Longhi: General methods for the assessment of disorder: PAGE, limited proteolysis, analytical size exclusion chromatography, CD, thermal shift assay, intrinsic fluorescence

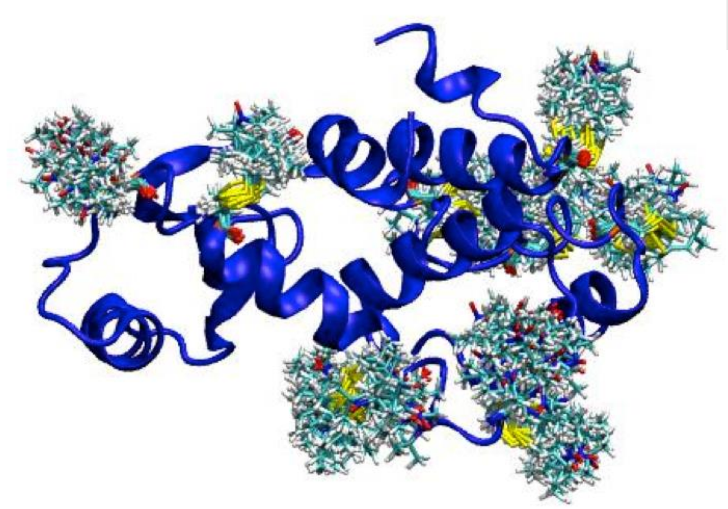
10:30 Tea & coffee (A11/205)

11:00 Lecture 13 Sonia Longhi: Advanced methods of disorder investigation: vibrational spectroscopy of cyanylated cysteine, Trp-Cys quenching, site-directed spin-label EPR spectroscopy

12:30 Lunch (A35 Atrium)

Computational methods

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



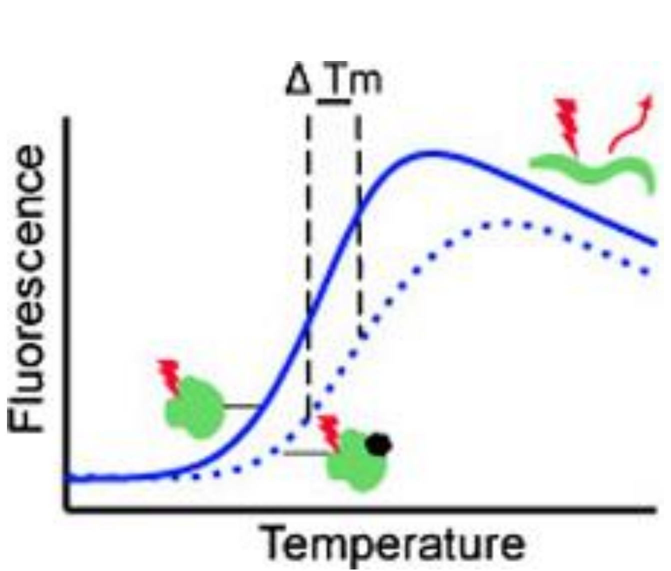
➢ Relatively cheap

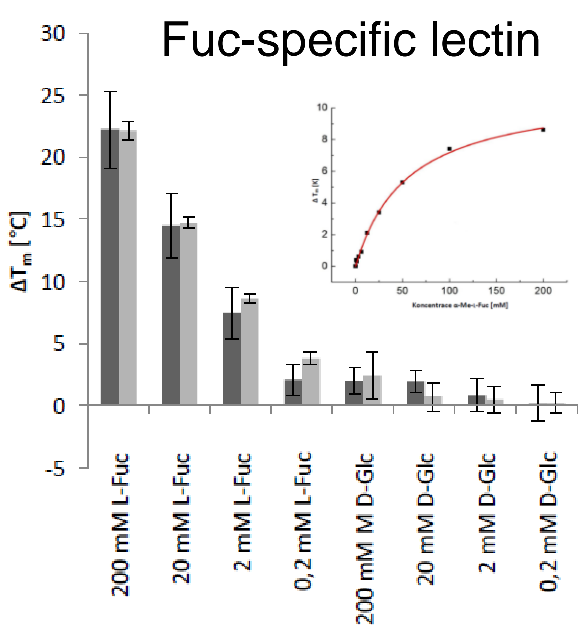
➢ Less accurate

➢ Ideally to be combined with experiment

Interaction analysis by stability

- 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



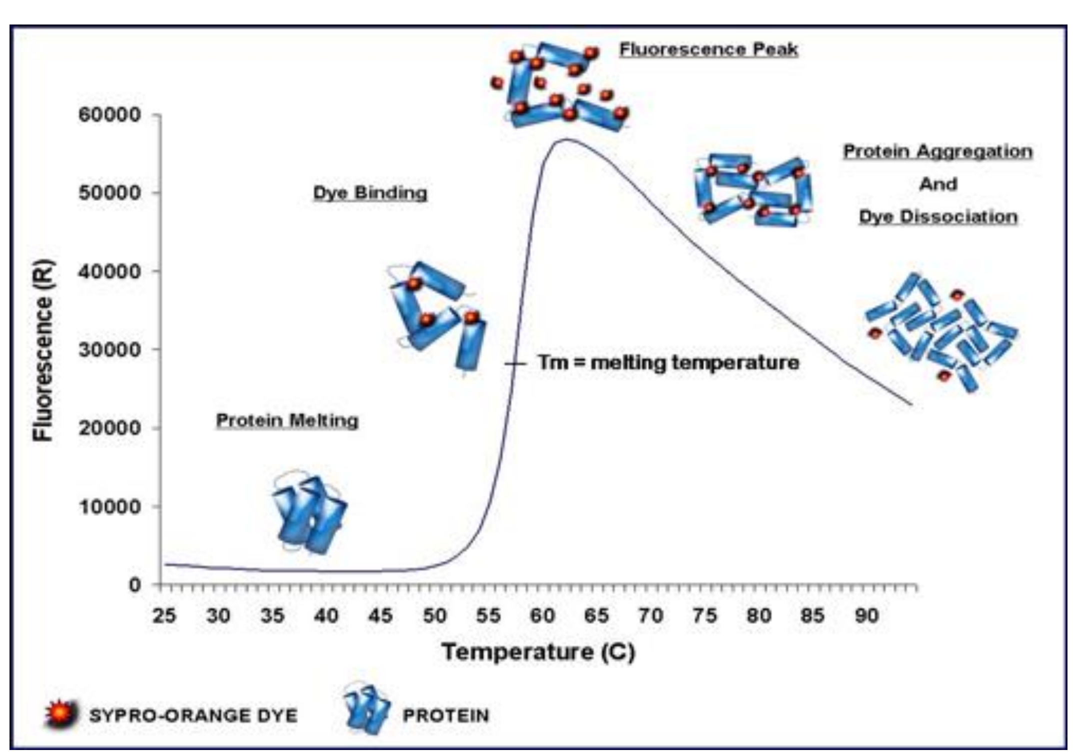


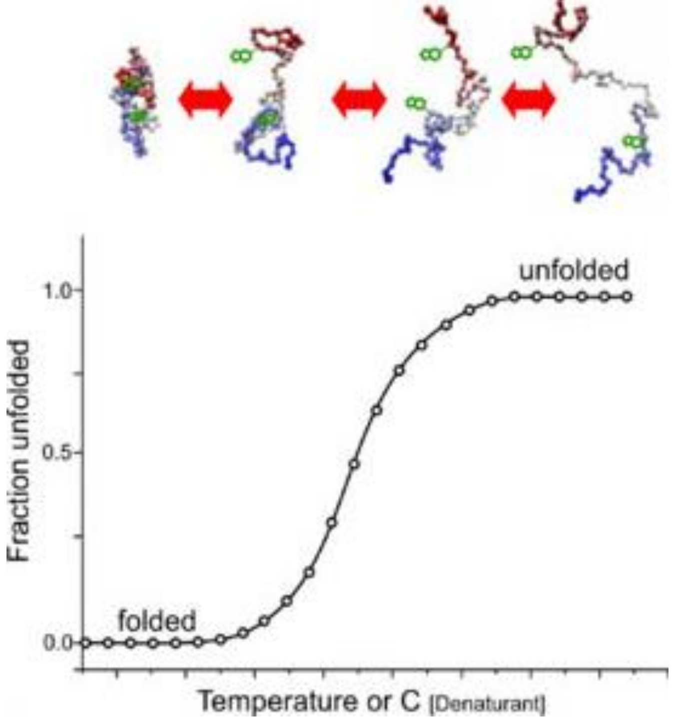
Thermal shift assay (TSA)

Differential scanning fluorimetry (DSF)

- Shift of fluorescence of external dye
- Change in intrinsic fluorescence (Trp)

(Semi-) Quantification of interaction





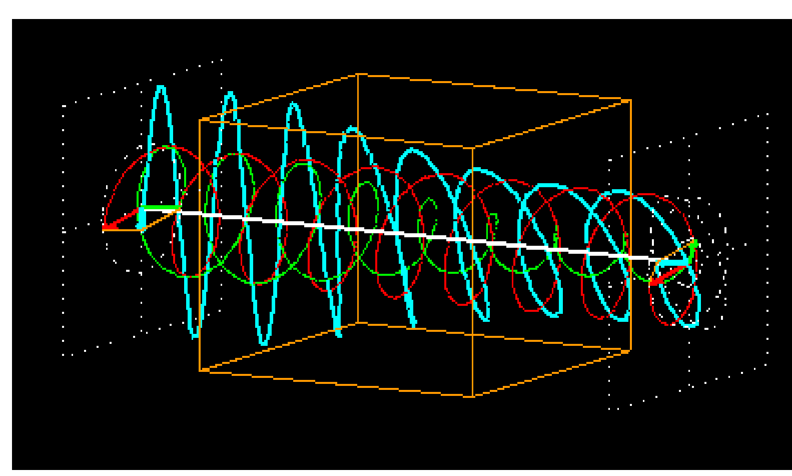
Thermal shift assay (TSA)

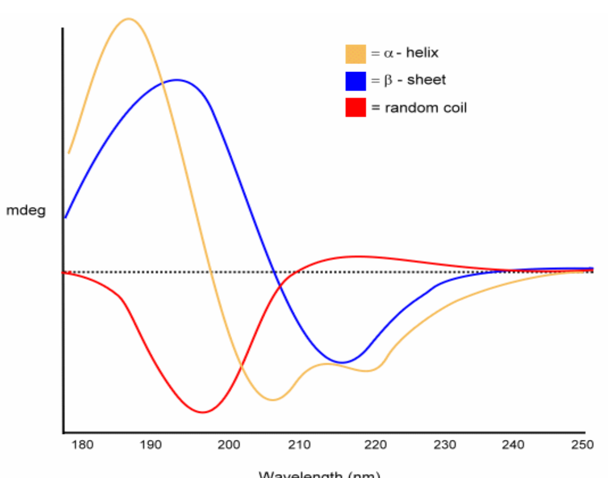
Advantages	Disadvantages
High-throuput	Qualitative/semiquantitative only
Broad range of interacting ligands applicable	Not suitable for protein-protein interactions (signal overlap)
	Interference with fluorescent dye/ Tryptophan presence needed

Circular dichroism spectroscopy (CD)

- Chiral compounds interact with circularly polarized light
- Proteins (and nucleic acids) are chiral
- Spectrum is **secondary structure specific**

⇒ Widely used for IDPs





CD spectra are additive – beware of **protein-protein interaction analysis !**

Fluorescence

Fluorescence intensity, Fluorescence Anisotropy (FA)

- Binding close to dye affects fluorescence – intensity, λ_{max}
- Binding of big molecule affects movement – change in FA

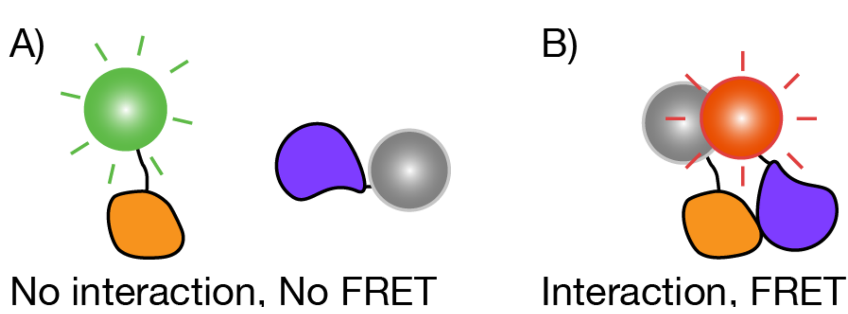
Int.
0.5
0.4
0.3
0.2
0.1
0
290 350 400 450 480
Wavelength [nm]

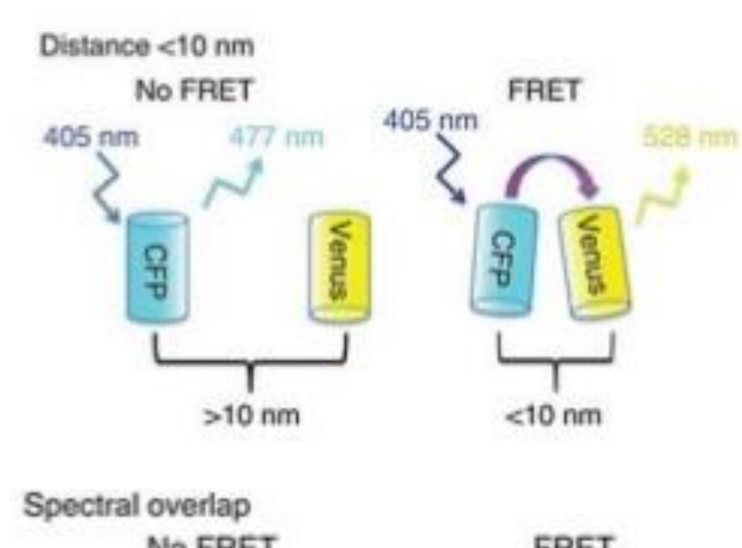
monochromator
Excitation light
Vertical polarizer
$I_{VV} \equiv I_{VH}$
r: small
$I_{VV} > I_{VH}$
r: large
Vertical and horizontal polarizer
monochromator
detector

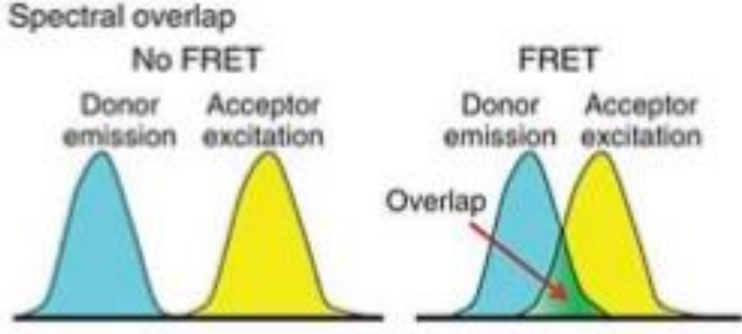
HO O O COOH
Fluorescein

Fluorescence Resonance Energy Transfer (FRET)

- Donor and acceptor molecules must be in close proximity (10-100 Å)
- Absorption spectrum of acceptor must overlap the fluorescence emission spectrum of the donor
- Donor absorption and emission spectra should have minimal overlap







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

Fluorescence base methods

Method	Advantages	Disadvantages	Area of application
Fluorescence Intensity	• Simple • Requires small quantities of protein, >10 nM of protein in the cuvette • Kd in the nM-mM range • Experiments can be done in rapid kinetic mode	• Suitability limited by changes in fluorescence between the free and bound molecule • Sensitive to inner filter effect, auto-fluorescence interference and photobleaching	• Protein-protein interaction • Protein-peptide interaction • Protein-nucleic acid interaction • Small molecule interaction
Fluorescence anisotropy	• Requires small quantities of protein, >10 nM of protein in the cuvette • Insensitive to inner filter effects and photobleaching • Suitable for small ligands (<10 kDa) • Kd in the nM-mM range • Experiments can be done in rapid kinetic mode	• Suitability limited by the lifetime of the dye, ligand size and change in molecular weight • Auto-fluorescence interference	• Protein-protein interaction • Protein-peptide interaction • Protein-nucleic acid interaction • Small molecule interaction
Fluorescence energy transfer (FRET)	• Simple • Suitable for inter- and intra-molecular distances (< 5 nm) • Wide range of fluorescent donors and acceptors • Experiments can be done in rapid kinetic mode	• Requires multiple fluorescent labels • Sensitive to inner filter effect, auto-fluorescence, homoFRET and photobleaching • Limited to short distances of interaction for high signal changes	• Protein-protein interaction • Protein-peptide interaction • Protein-nucleic acid interaction

Gijsbers A. *et al* **2016** Fluorescence Anisotropy as a Tool to Study Protein-protein Interactions. *J Vis Exp*

Microscale thermophoresis (MST)

- Particle motion in temperature gradient
- Sensitive to **size**, **hydration shell** and **charge**

Molecules

Initial State

Temp. Jump

Thermo-phoresis

Steady State

Back-diffusion

Fluorescence

10 20 30 40 50

IR-Laser on

Time [s]

IR-Laser off

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

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

size

hydration shell

charge [2]

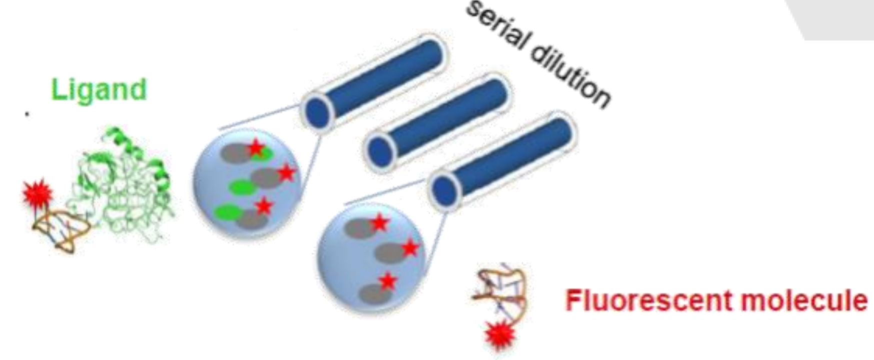
+

-

MST – Basic principles

Labeled molecule A

Dilution series of molecule B



1000
900
800
700
F_{norm}
IR-laser on
bound
unbound
IR-laser off
0
10
20
30
40
Time (s)

Saturation
20
15
10
5
0
ΔF_{norm} (‰)
K_d
Baseline
10^{-4}
10^{-3}
0.01
0.1
1
10
Ligand (nM)

Microscale thermophoresis (MST)

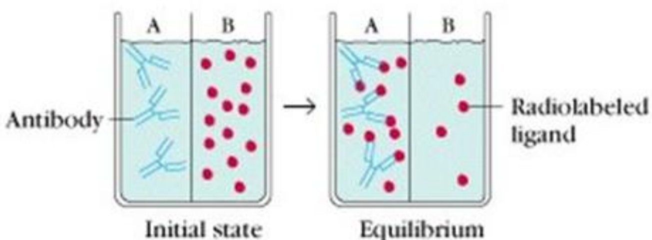
Advantages	Disadvantages
Sample concentration (pM/nM) and small volume	Labeling needed
No limitation on molecular size or molecular weight	Buffer conditions need to be stable
No immobilization	Conformational changes induced by IR-laser heating may be problematic
Broad buffer compatibility, complex environment possible	

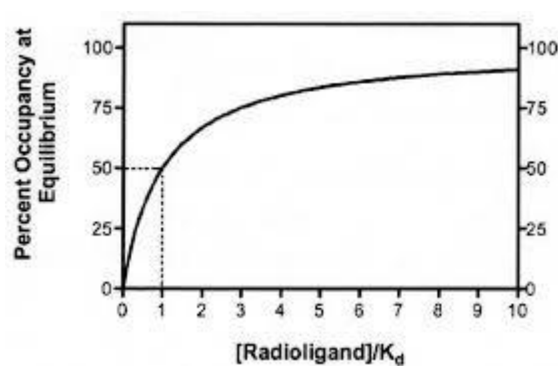
Equilibrium dialysis

- Protein and ligand solution is separated by membrane with $MW_{(ligand)} < MW_{CO} < MW_{(protein)}$

- Ligand final concentration measured after reaching equilibrium for different initial concentrations

- Data analyzed to determine K_D (Scatchard plot, non-linear analysis)

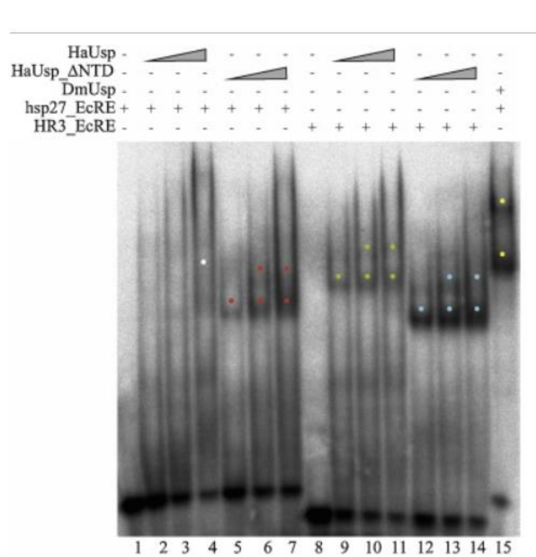




Electrophoresis

Affinity capillary electrophoresis (ACE)
Electromobility shift assay (EMSA)

- Electrophoresis used to distinguish free and bound form of protein
- Shift in mobility due to change in the charge:size ratio
- In gel, in capillaries
- K_D can be calculated

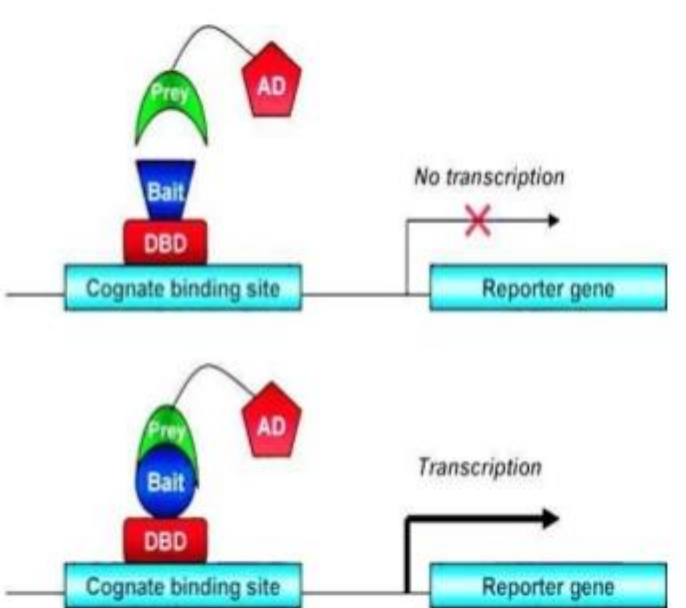


Protein interaction with DNA by EMSA
Wycisk 2018 J Steroid Biochem

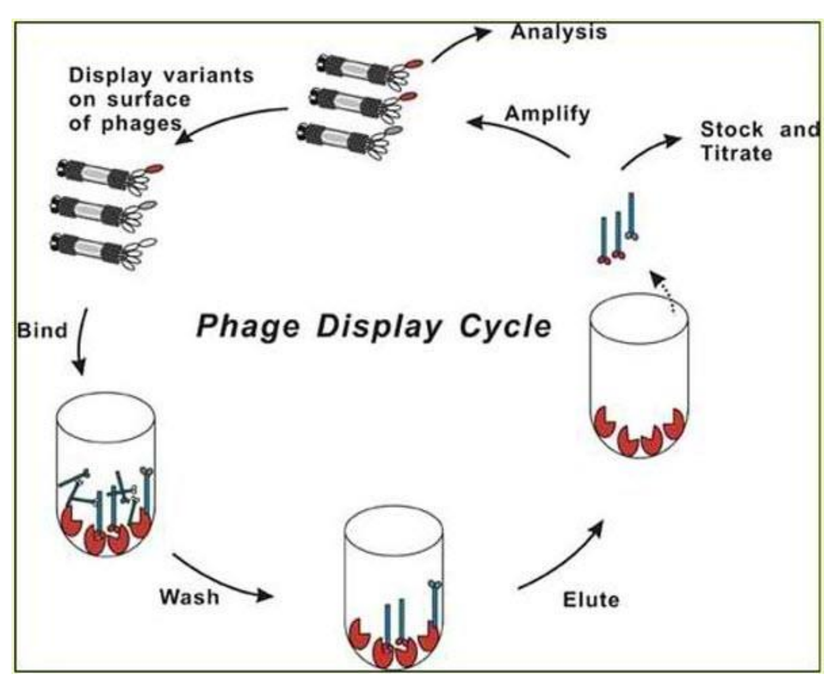
Complex techniques

- Indirect detection of molecular interaction
- Multi-step approaches

Yeast two-hybrid (Y2H)



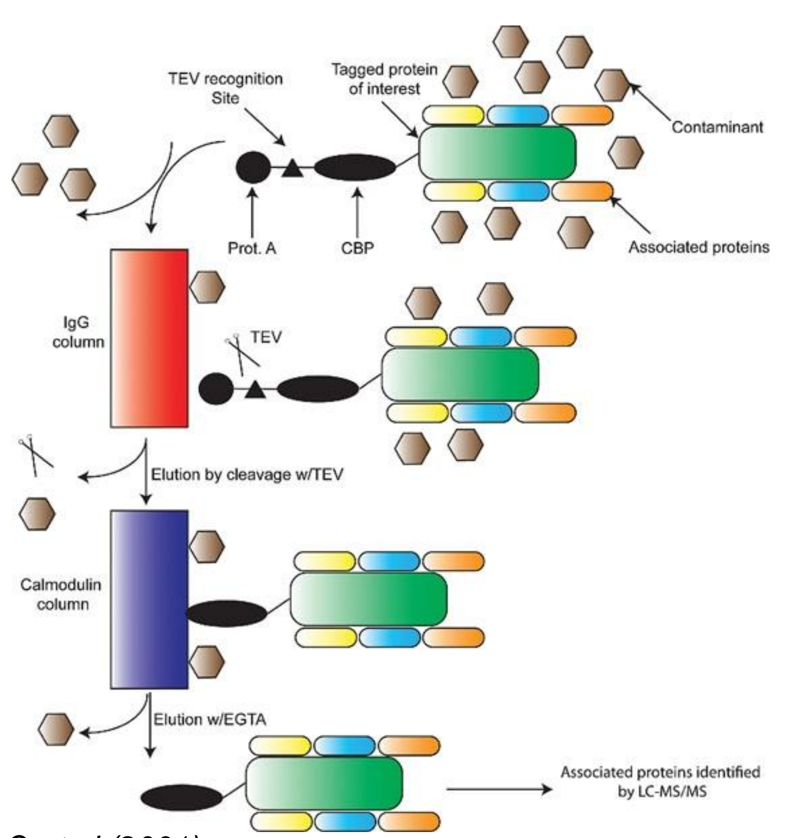
Phage display



Complex techniques

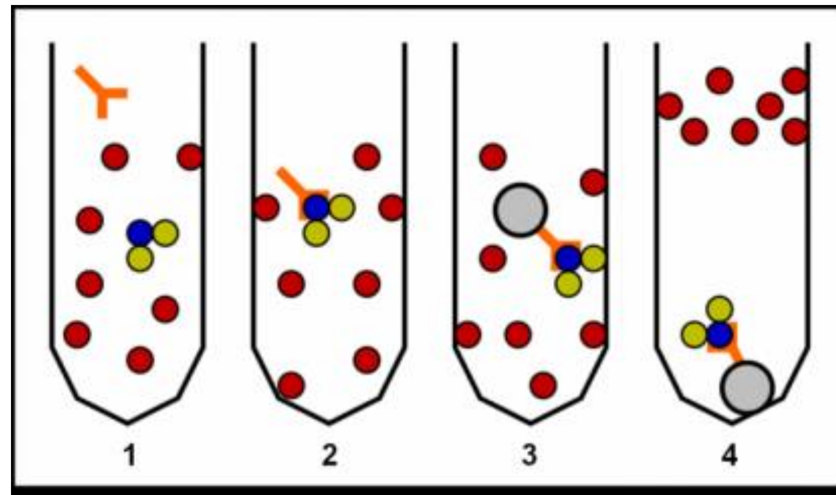
Pull-down assay

Tandem affinity purification



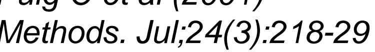
Puig O et al (2001)
Methods. Jul;24(3):218-29

Co-immunoprecipitation



[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.

MS analysis

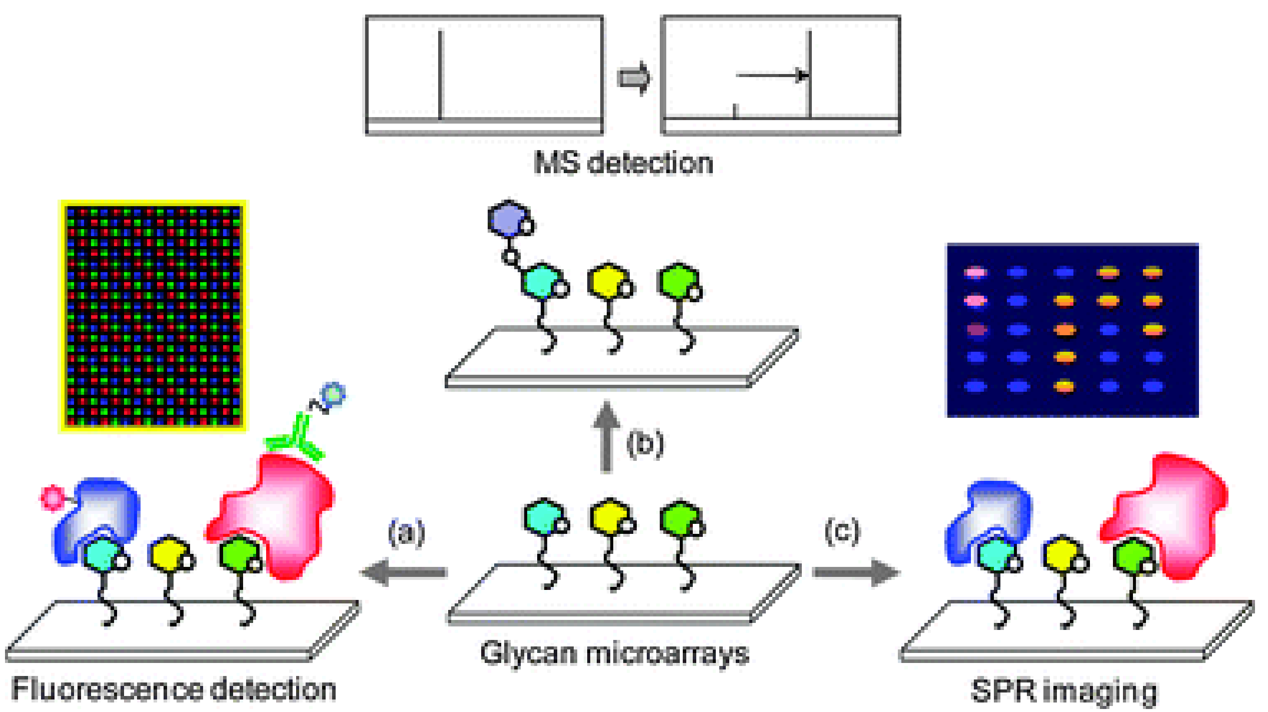


Complex techniques

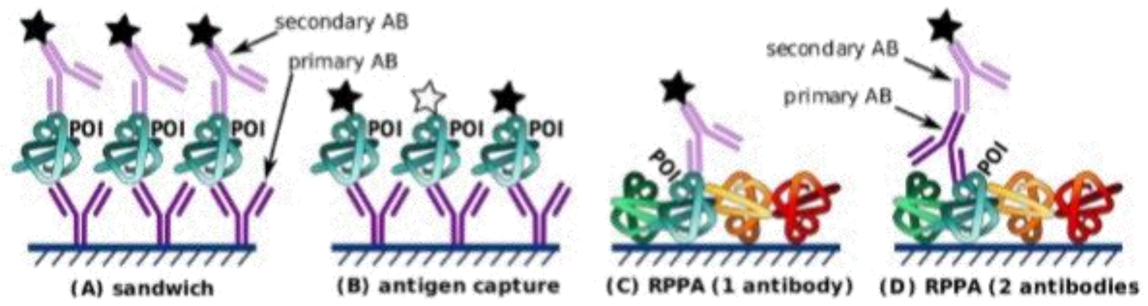
Advantages	Disadvantages
Utilize biological systems	Utilize biological systems
Identification of complicated complexes components possible	Time-demanding

Microarrays

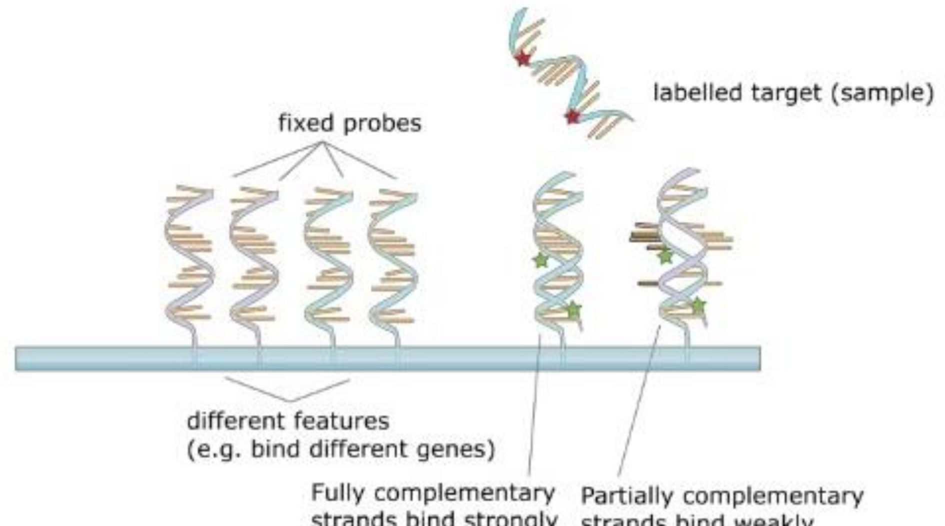
- High screening capacity possible
- Semi-quantitative



Microarrays



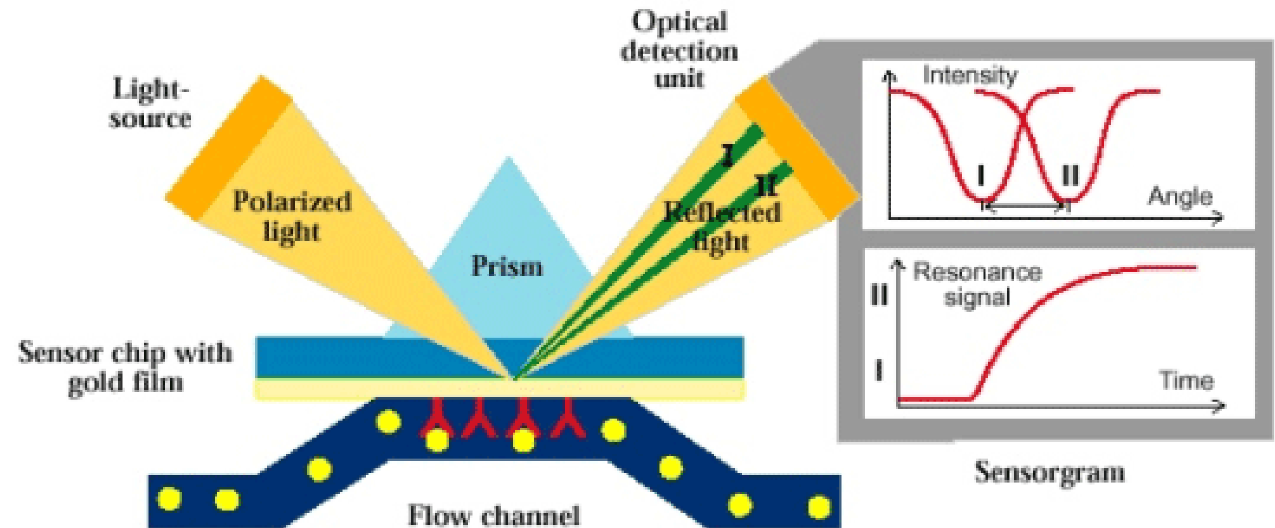
- Various immobilized molecules
  (protein, nucleic acid, saccharide)



Surface plasmon resonance (SPR)

- Detection of molecular interaction on a chip surface

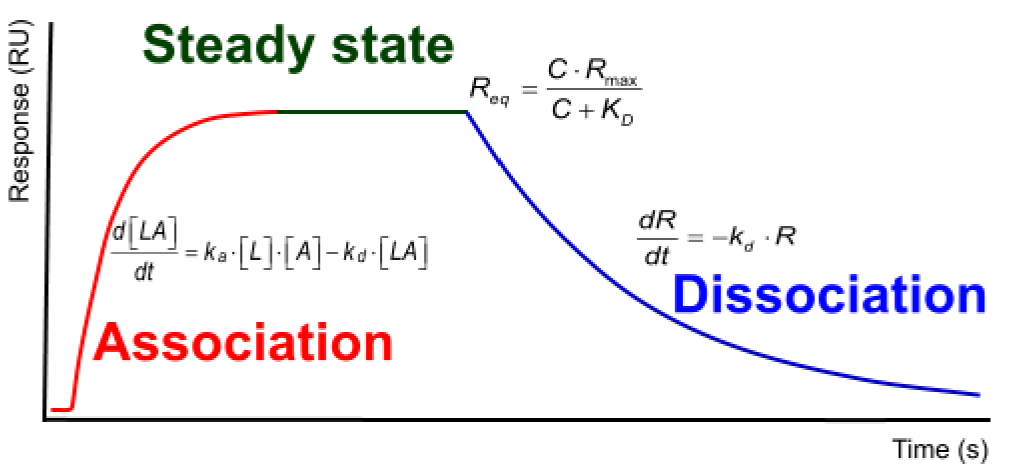
- Various set-ups:
  protein-protein
  protein-nucleic acid
  protein-cell/virus
  protein-ligand
  protein-lipid membrane

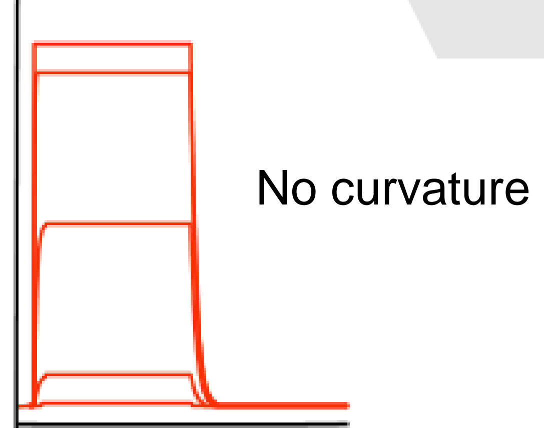


Surface plasmon resonance (SPR)

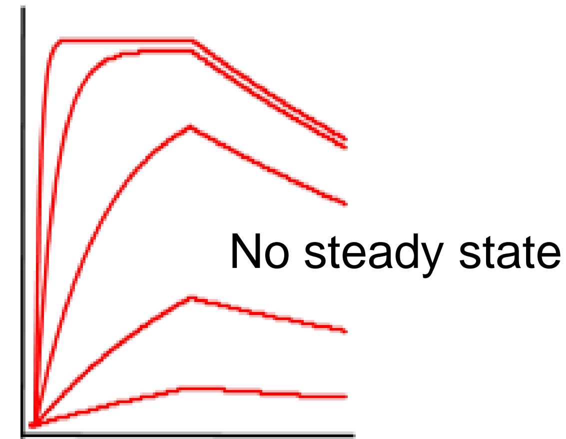
- Typical binding curve – association and dissociation phase, (surface regeneration)

SPR – affinity vs. kinetics





- **Steady state** only (quick association/dissociation) – **only K_D**



- Kinetic measurement

  $K_D = k_d / k_a$

Same affinity but different kinetics

- 4 compounds with the same affinity $K_D = 10\ \text{nM} = 10^{-8}\ \text{M}$
- The binding kinetic constants vary by 4 orders of magnitude

Concentration = 100 nM

Concentration = 1000 nM

k_{on} $M^{-1}s^{-1}$	k_{off} s^{-1}
10^6	10^{-2}
10^5	10^{-3}
10^4	10^{-4}
10^3	10^{-5}

Completely blocked target - all target sites occupied

Response

Time

Time

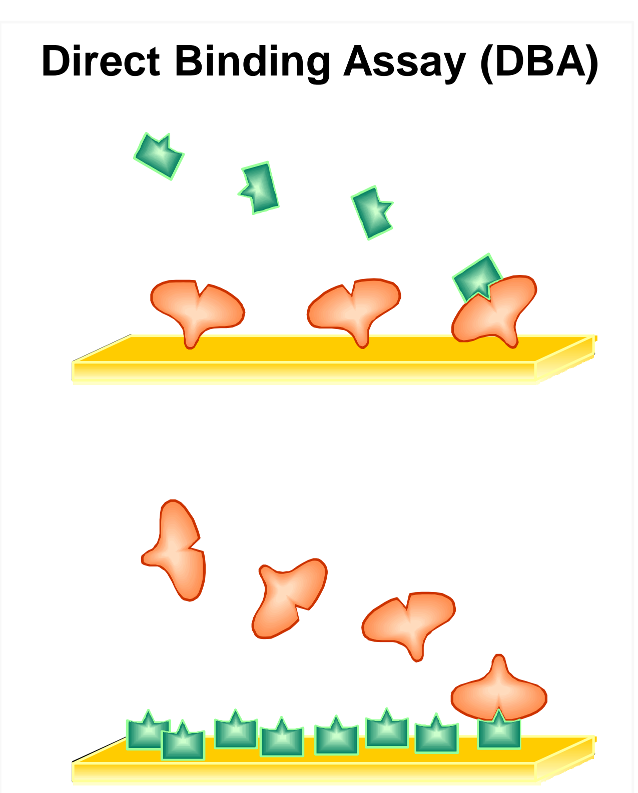
Compounds with slow off-rates occupy the target for a longer time

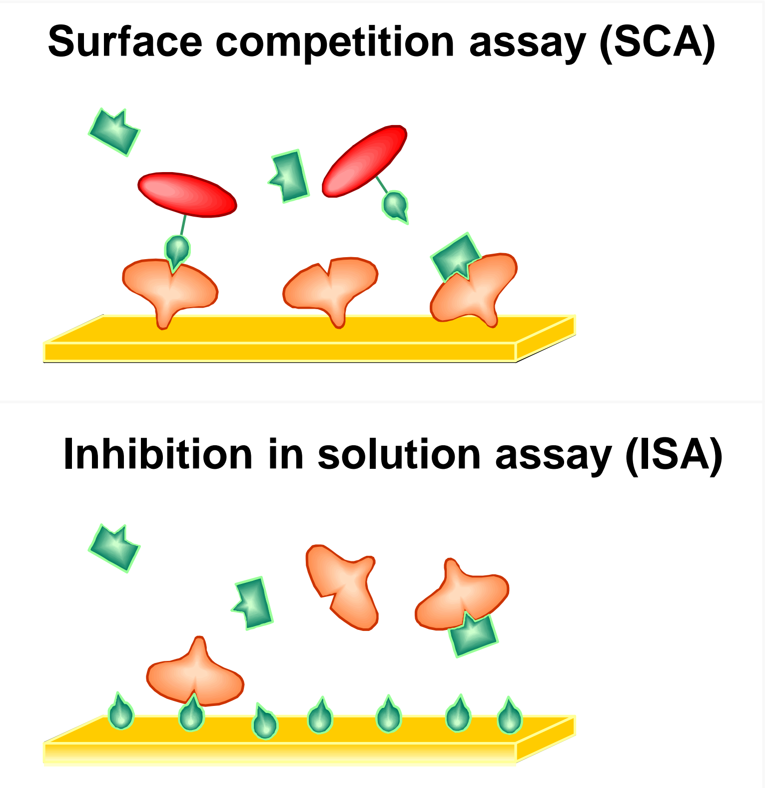
Flexibility in Assay Design

Multiple assay formats providing complementary data

Direct measurement

Indirect measurement



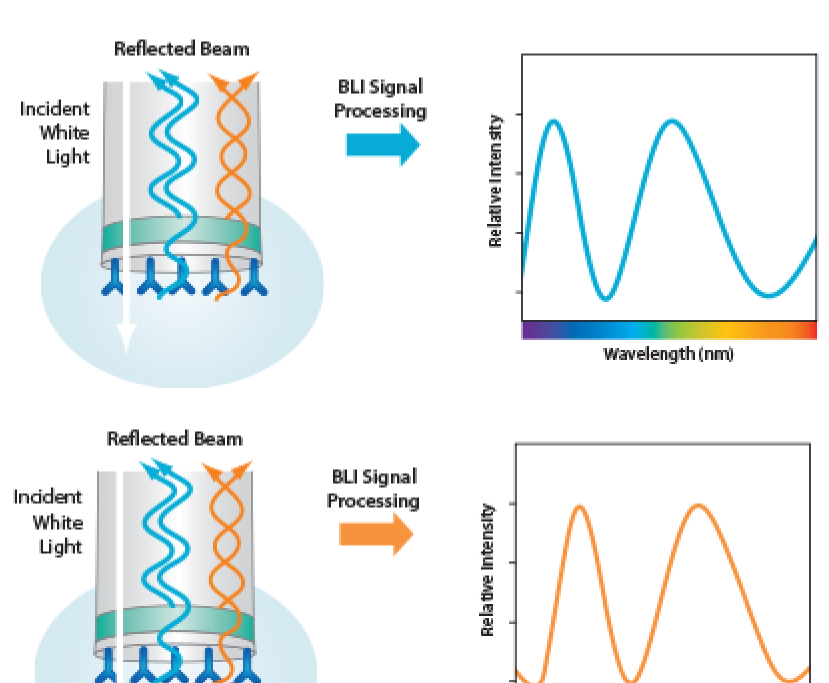


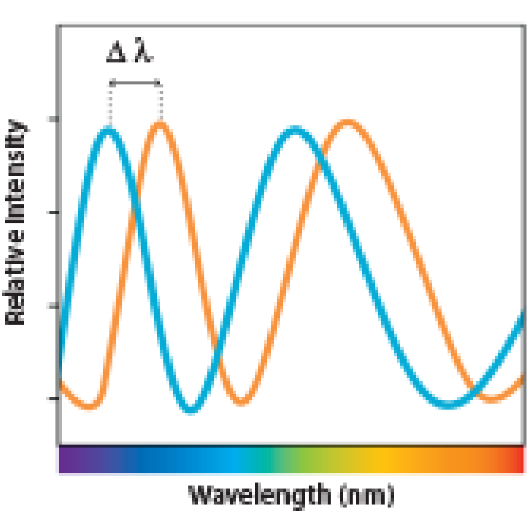
Surface plasmon resonance (SPR)

Advantages	Disadvantages
No labeling	Immobilization needed (potential binding site obstruction, interaction with matrix, avidity effects)
Low sample consumption	Signal affected by buffer mismatch
Real time assay (kinetics)	
Sensitivity	
No molecular size limit	

Biolayer interferometry (BLI)

- Detection of molecular interaction **on a surface**
- Light interference between reflexion from ligand-exposed surface and internal reference layer

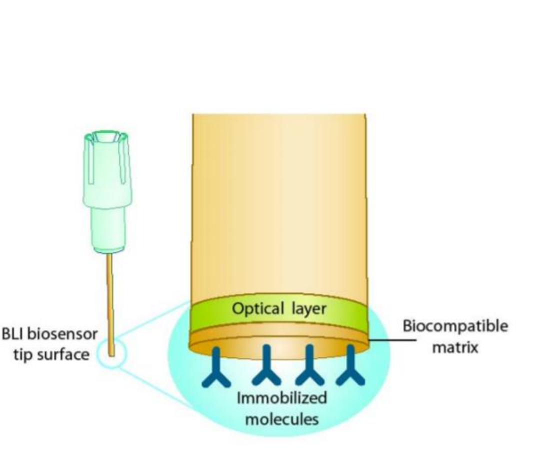


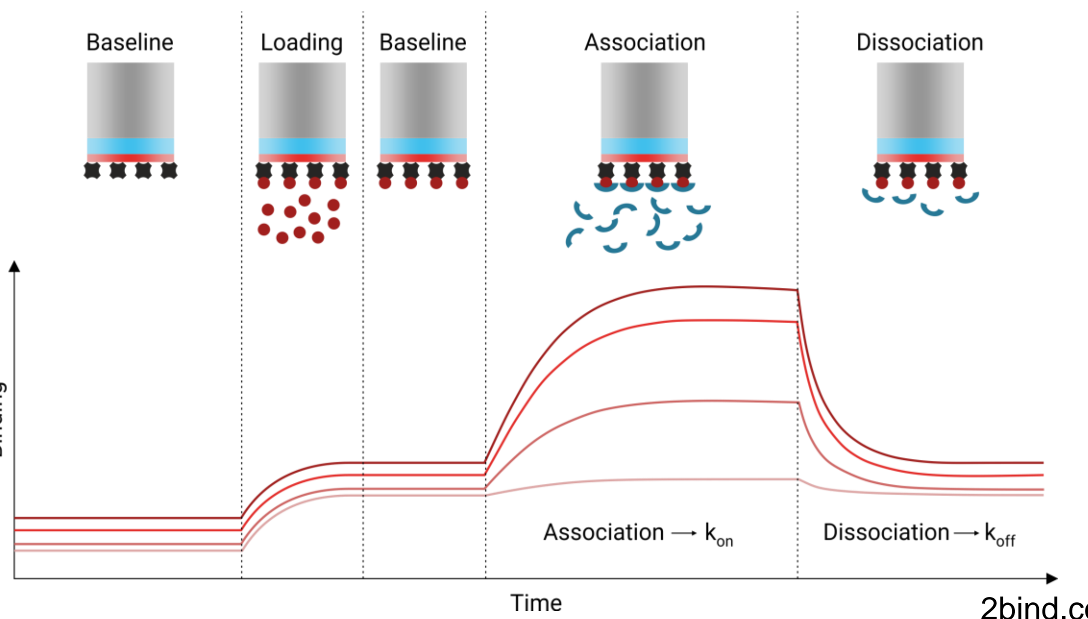


fortebio.com

Biolayer interferometry (BLI)

- Signal depends on thickness of surface layer
  = low signal for small molecules
- Real-time measurement (kinetics)
- Measurement in complex samples possible (cell lysate, blood serum, juice)





2bind.com

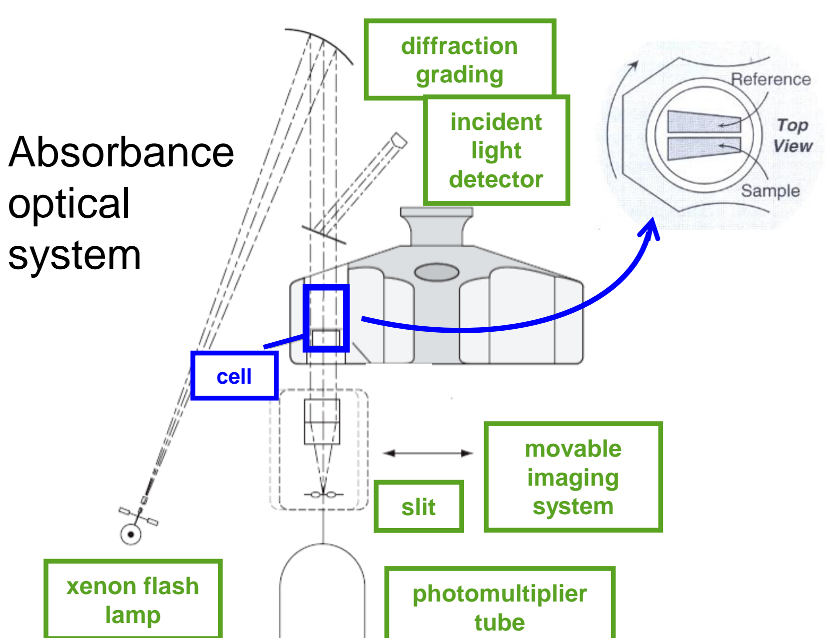
Biolayer interferometry (BLI)

Advantages	Disadvantages
No labeling	Immobilization needed (potential binding site obstruction, interaction with matrix, avidity effects)
Low sample consumption	Low sensitivity for small molecules
Real time assay (kinetics)	
Complex samples	

Analytical ultracentrifugation (AUC)

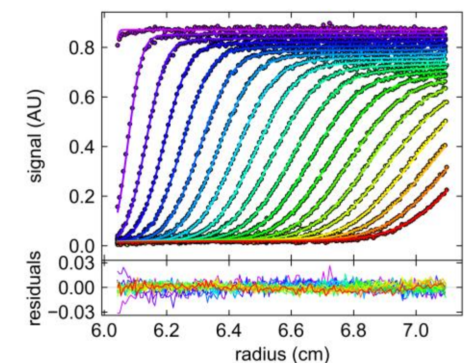
- Particle analysis in centrifugal field
- First-principle method – no calibration, no labeling required
- Study of molecules directly in solution
  – possibility to vary buffer conditions

Absorbance optical system

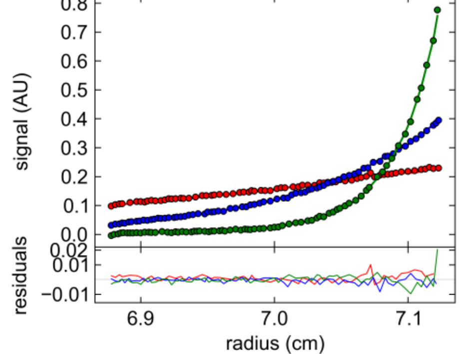


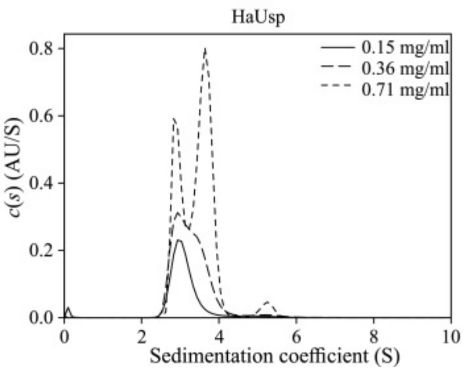
Analytical ultracentrifugation (AUC)

- **Two modes**
  - Sedimentation velocity (SV)
  - Sedimentation equilibrium (SE)



- Used for:
  - Particle size, MW
  - Oligomeric studies
  - Sample homogeneity
  - Interaction analysis





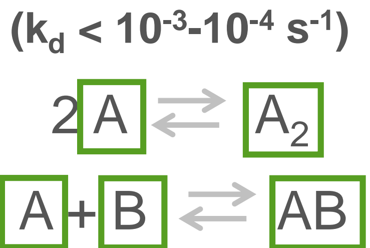
HaUsp Protein dimerization
Wycisk 2018 J Steroid Biochem

Reaction kinetics in SV

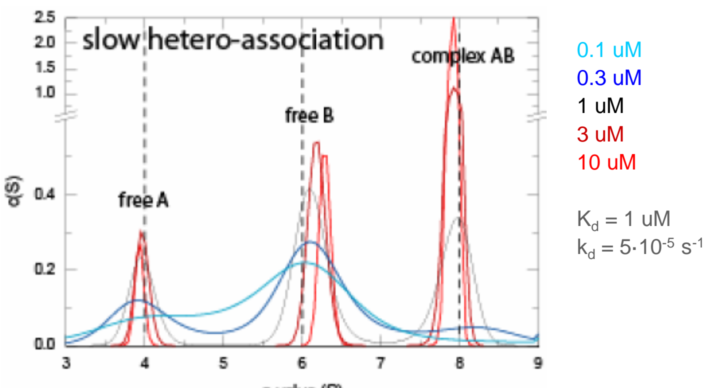
Sedimentation depends on the life time of the complexes relative to the time-scale of SV experiment.

SLOW INTERACTIONS

($k_d < 10^{-3}$-10^{-4} s^{-1})



Sedimenting species stable, peak positions constant, relative peak areas change with increasing concentration



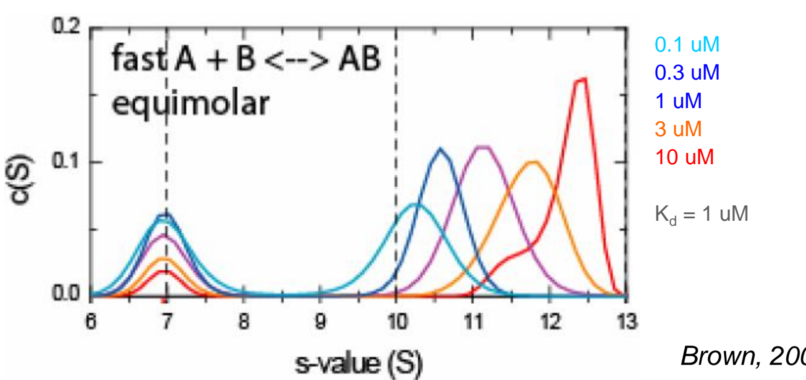
FAST INTERACTIONS

($k_d > 10^{-3}$ s^{-1})

2 A ⇄ A_2

A + B ⇄ AB

Rapid interconversion between complex and free species, peak position change with increasing concentration



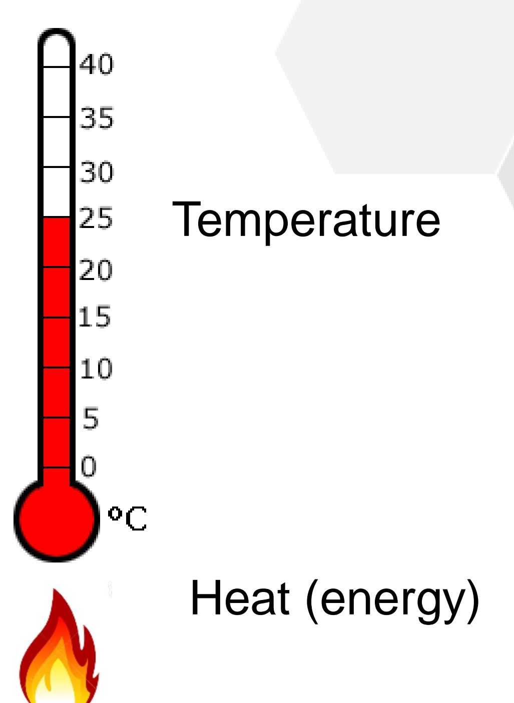
Brown, 2008

Analytical ultracentrifugation (AUC)

Advantages	Disadvantages
No labeling	Time-consuming
In-solution technique	Higher sample consumption
Applicable to self-associating systems	Not suitable for small molecules
	Higher expertise needed

Calorimetry

Calorimetry



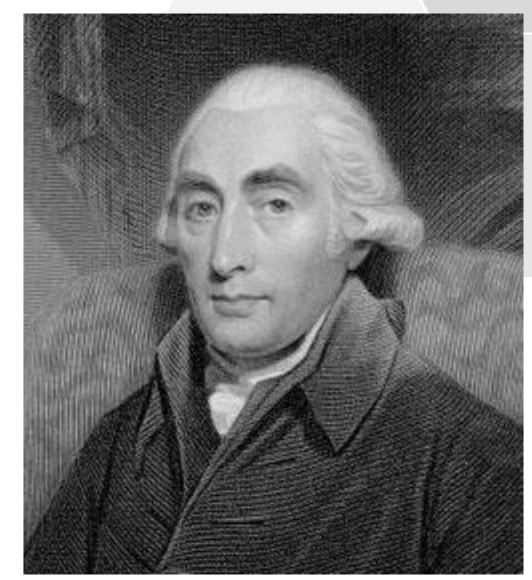
Heat ≠ Temperature

- **Calorimetry**
  - Latin *calor* – heat

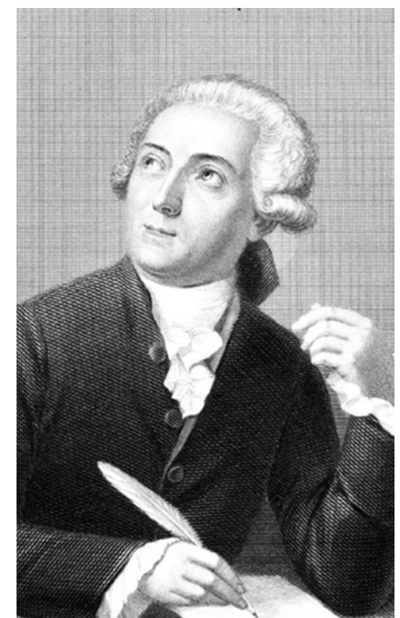
    Greek *μέτρον* – to measure
  - thermodynamic technique based on measurement of heat that may be generated (exothermic process) or consumed (endothermic process) by sample

- **Calorimeter**
  - instrument for measuring the quantity of heat released or absorbed in process of chemical reaction

History of calorimetry: „Founding Fathers“



- **Joseph Black (1728 – 1799)**
  - „founder of the calorimetry“
  - first who recognize the distinction between heat and temperature



- **Antoine Lavoisier (1743 – 1794**)
- **Pierre-Simon Laplace (1749 – 1827)**

History of calorimetry: First calorimeter

Air in

Air out

The guinea pig was placed at rest in a chamber surrounded by ice.

A second ice-filled chamber insulated the inner chamber from the environment.

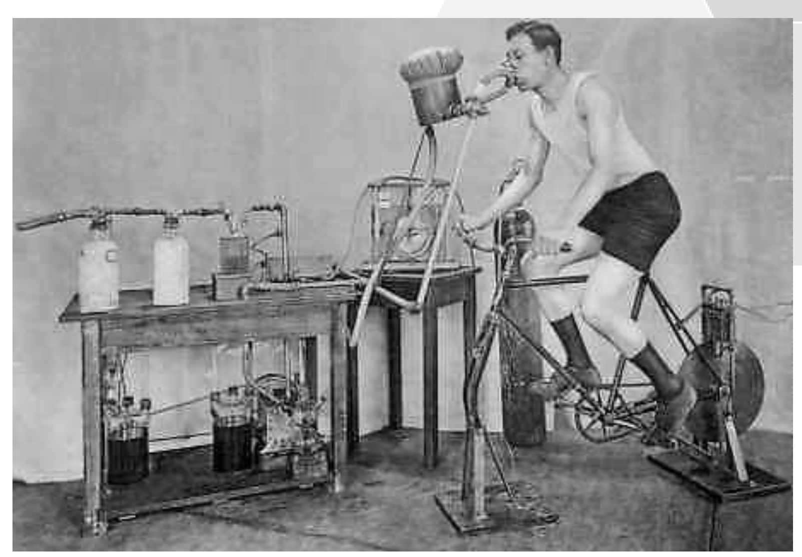
Outer ice-filled chamber

Inner ice-filled chamber

Collection port from inner chamber

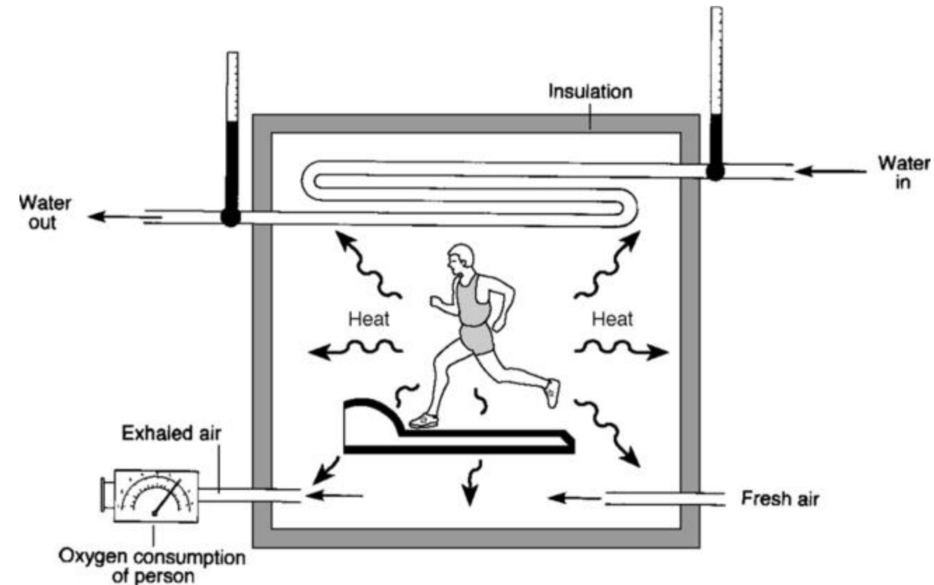
As the animal's body heat is conducted to the ice of the inner chamber, the ice melts, producing water. The volume of water is proportional to the amount of heat produced.

Calorimetry



- **INDIRECT CALORIMETRY** –

  calculates the heat generated by living organism when their metabolic processes yield waste carbon dioxide



- **DIRECT CALORIMETRY** –

  measures heat generated by living organism by placing the entire organism inside the calorimeter for the measurement

Calorimetry units

- 1 **calorie** = 4.184 **Joules**
- 1 *calorie* = energy needed to raise the temperature of 1 g of water by 1°C (at 1 atm)
- 1 *Joule* = energy needed to apply force of 1 N over a distance of 1 m

- *10 000 calories needed to heat 500g water from 10° to 30°*

Drink cold beer to loose weight!

Slide by Arthur Sedivy, VBCF

Calorimetry units

In nutrition kcal are usually referred to as “calories” but they are actually kilocalories!

Neglect a factor of 1000!

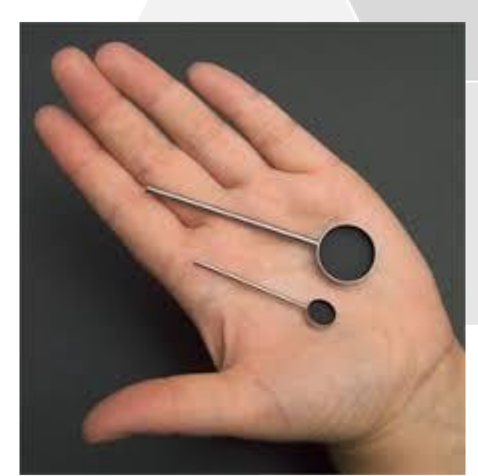
energy			fat				carbohydrates				fibre	protein		salt	
kJ	kcal		total		of which saturates		total		of which sugars						
per portion	per portion	%RI* (Adult)	per portion (g)	%RI (Adult)	per portion (g)	%RI (Adult)	per portion (g)	%RI (Adult)	per portion (g)	%RI (Adult)	per portion (g)	per portion (g)	%RI (Adult)	per portion (g)	%RI (Adult)
1262	301	15	12	18	6.0	30	31	12	7.3	8	2.4	16	31	1.6	27

- 1 calorie = 4.184 J
  1 Calorie= 1 kcal = 4184 J
  1 J = 0.000239 kcal = 0.2390 cal

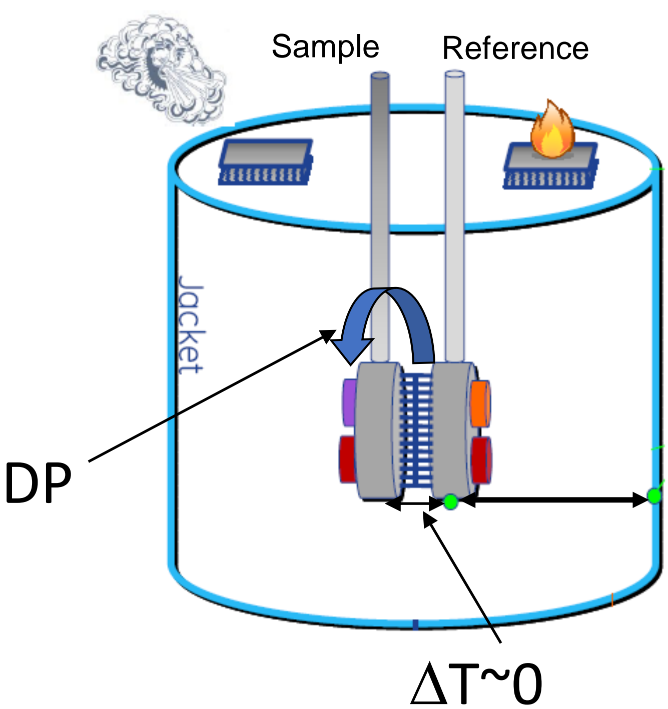
Slide by Arthur Sedivy, VBCF

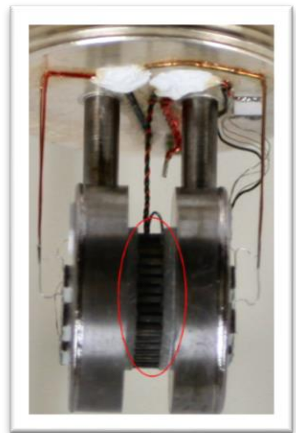
Microcalorimetry

- Limited sample amount – miniaturization



V_{cell} = 200 ul





The DP is a measured power differential between the reference and sample cells to maintain a zero temperature between the cells

Reference Calibration Heater
Sample Calibration Heater
Cell Main Heater

DP = Differential power
ΔT = Temperature difference

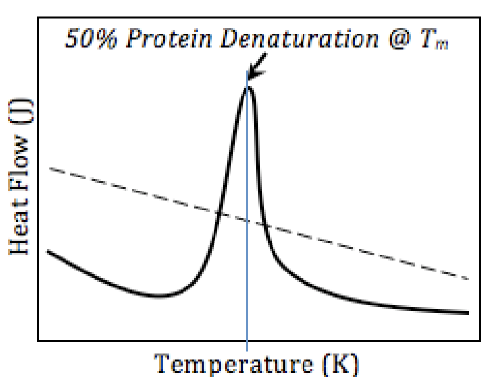
Microcalorimetry

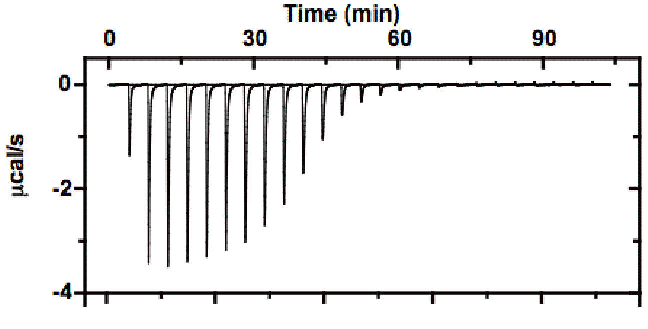
Differential scanning calorimetry – DSC

- Biomolecular stability in solution
- Provides insights into mechanisms of unfolding and refolding
- Midpoint **(T_m)** determination

Isothermal titration calorimetry – ITC

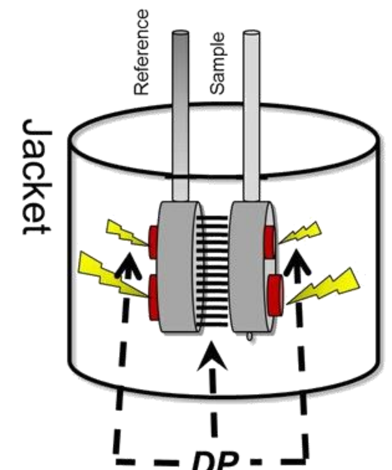
- Heat is released or absorbed as a result of the redistribution and formation of non- covalent bonds when the interacting molecules go from the free to the bound state.



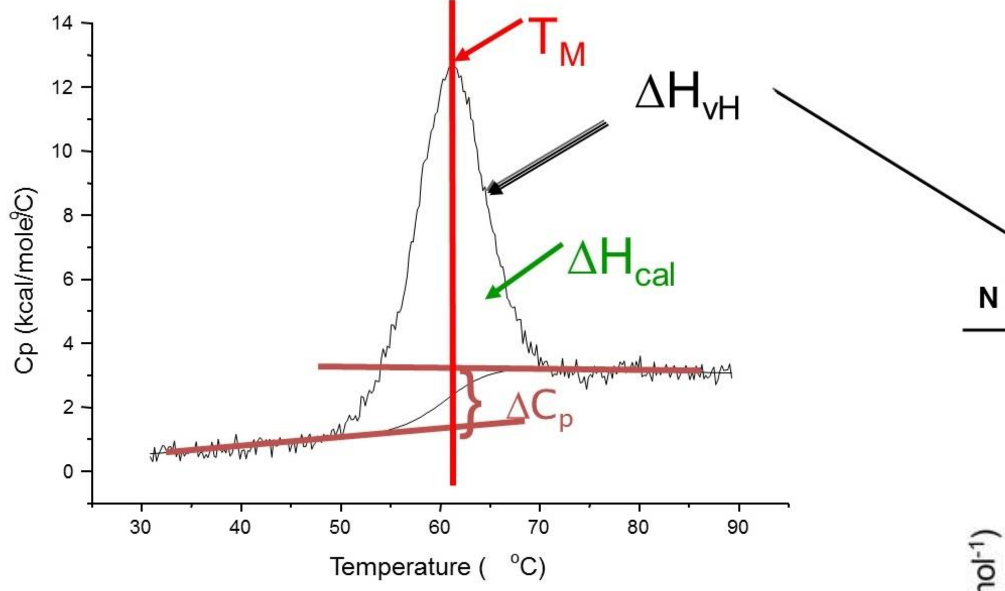


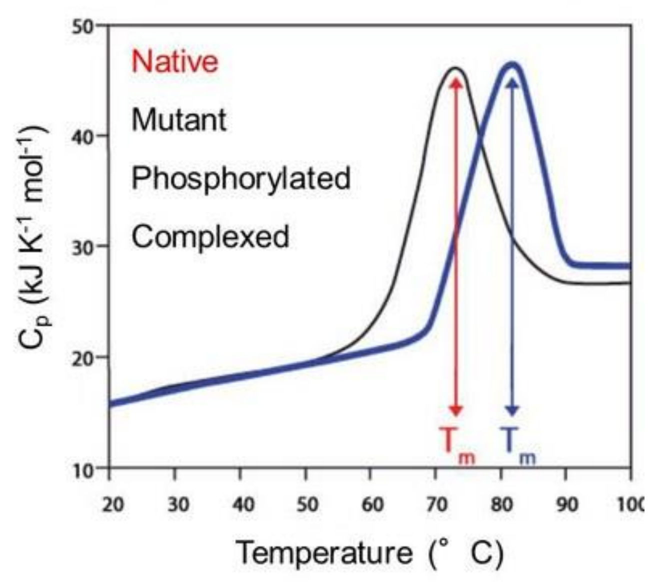
Differential scanning calorimetry (DSC)

- Measures the heat capacity in range of temperatures
- Ligand binds preferentially to native state of protein ⇒ complex denature at higher temperature
- Degree of stabilization depends on binding energy – comparison of complex and free protein allows to estimate binding energy



Differential scanning calorimetry (DSC)





- Peak area – calorimetric enthalpy ΔH_{cal}
  Total amount of protein
- Peak shape – van't Hoff enthalpy ΔH_{vH}
  Cooperativity of transition

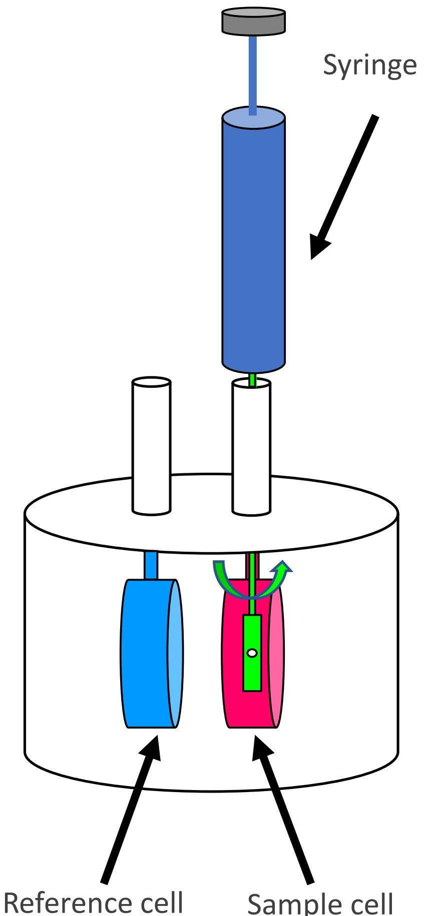
Differential scanning calorimetry (DSC)

Advantages	Disadvantages
No labeling	Useful only for tight binding with very slow equilibration
In-solution technique	Higher sample consumption
Gives information on the nature of binding event	Sensitivity depends on many parameters

Isothermal titration calorimetry (ITC)

- “Ligand” in syringe (also serves as mixing device)
- “Macromolecule” in sample cell

- Reverse arrangement possible
- Reference cell filled with water



Performing an ITC assay

0.5
0.0
−0.5
−1.0
−1.5
−2.0
−2.5

µcal/sec

0 20 40 60 80 100 120 140 160 180 200 220

Time (min)

The titration data

Time (min)

0 30 60 90 120

0

-3.0

-6.0

μcal/sec

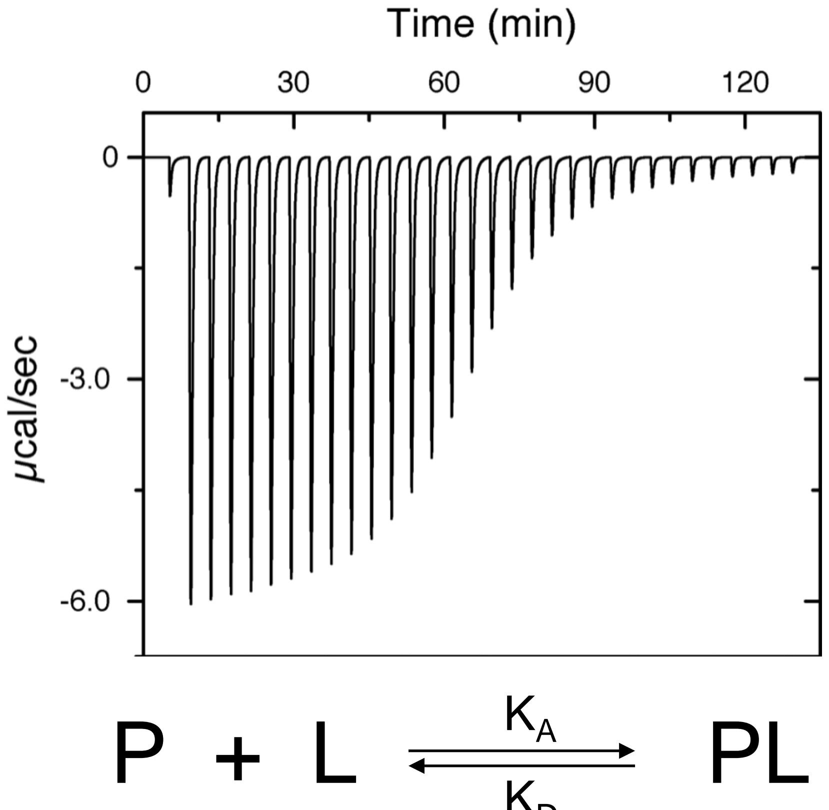
Raw ITC data is a measure of the power difference supplied to each cell

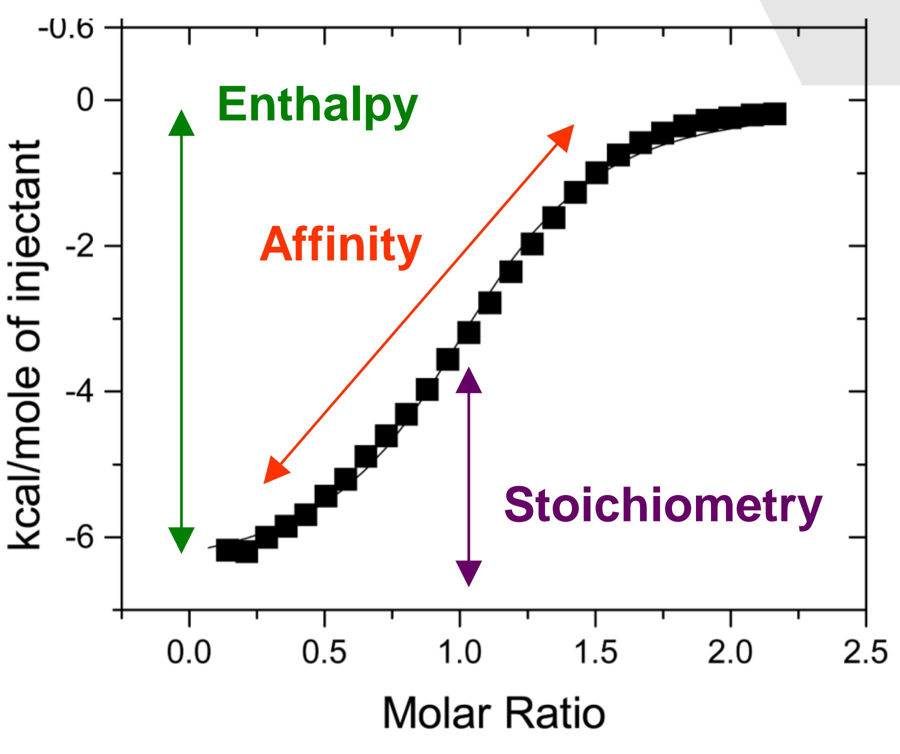
0.6-6 μcal/sec

2.4 kW

0.6 kcal/sec

Peak integration



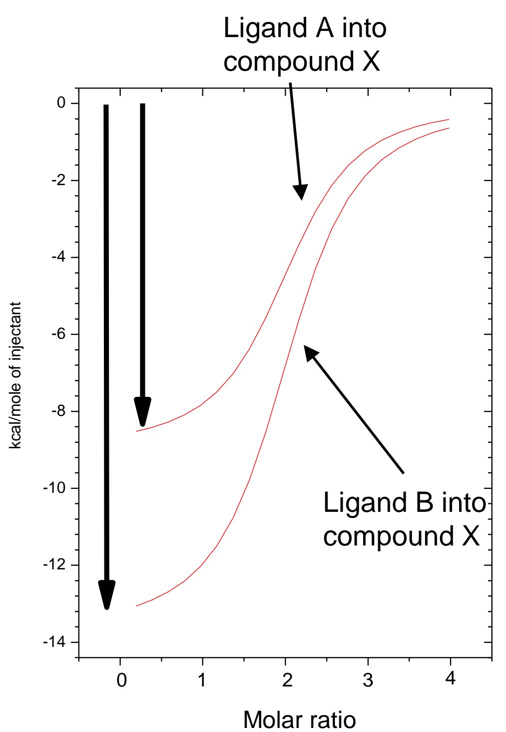


$$P + L \underset{K_D}{\overset{K_A}{\rightleftarrows}} PL$$

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

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

The energetics

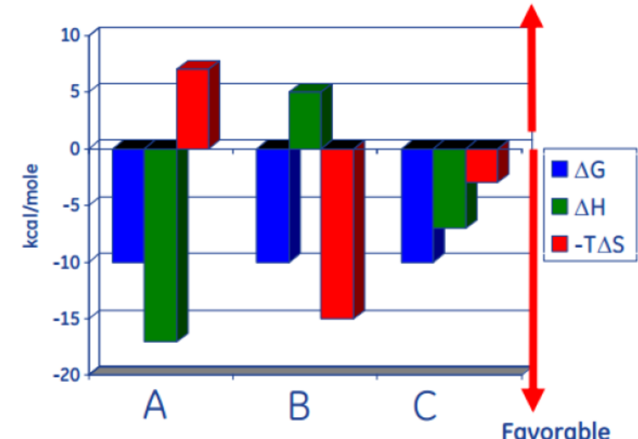


Same affinity, different energetics!
All three interactions have the same binding energy (ΔG)

A. Good hydrogen bonding with unfavorable conformational change

B. Binding dominated by hydrophobic interaction

C. Favorable hydrogen bonds and hydrophobic interaction



ITC results are used to get insights into mechanism of binding

Shape of the curve – „c value“

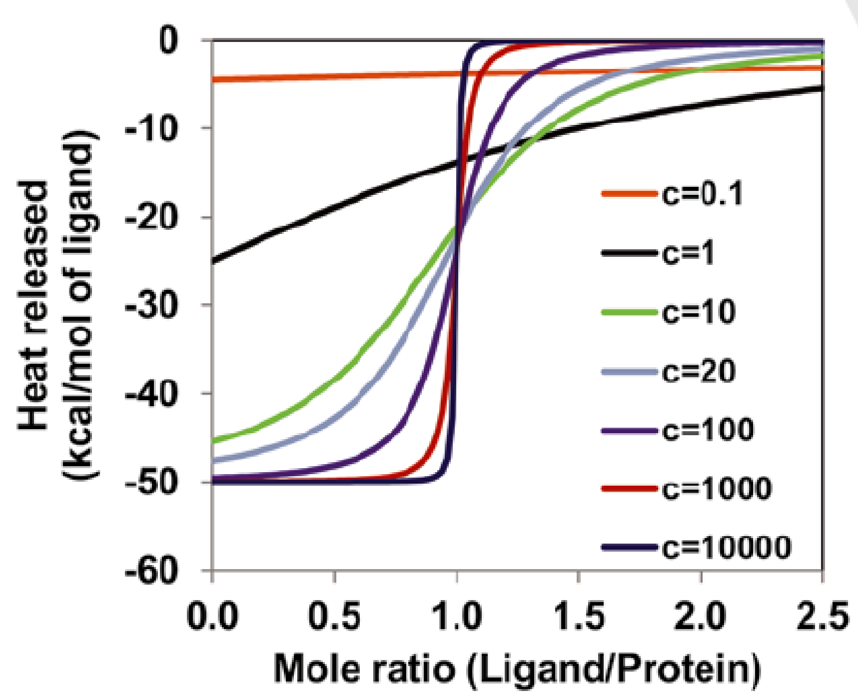
$c = [Protein]/K_D$

c = 10-100 Great

c = 5-500 Good

c = 1-5 and 500-1000 OK

c = < 1 and > 1000
competition ITC



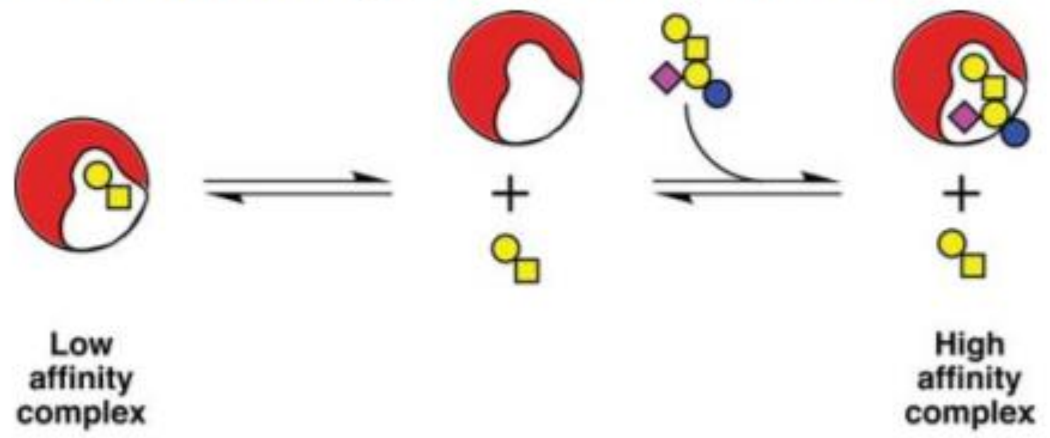
Low affinity ⇒ High sample consumption

⇒ **Imprecise** (or impossible) determination of **N**

High affinity ⇒ Low concentration of sample = Low sensitivity

⇒ **Imprecise** determination of $\mathbf{K_D}$

Competition titration



- High affinity ligand added to a solution of the low affinity complex
- High affinity ligand displaces the low affinity ligand
- Change in the apparent affinity and apparent enthalpy
- If parameters for one ligand are known, possible to calculate for the other ligand

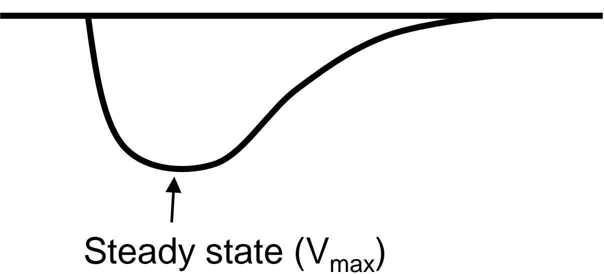
Single injection method

Whole used volume injected in single “burst”

- **Faster** (20 min/experiment)
- **Semi-quantitative** – imprecise parameter determination
- Applied for: fast screening

  unstable samples

  enzyme kinetics



Sample quality for ITC

Garbage IN – Garbage OUT !

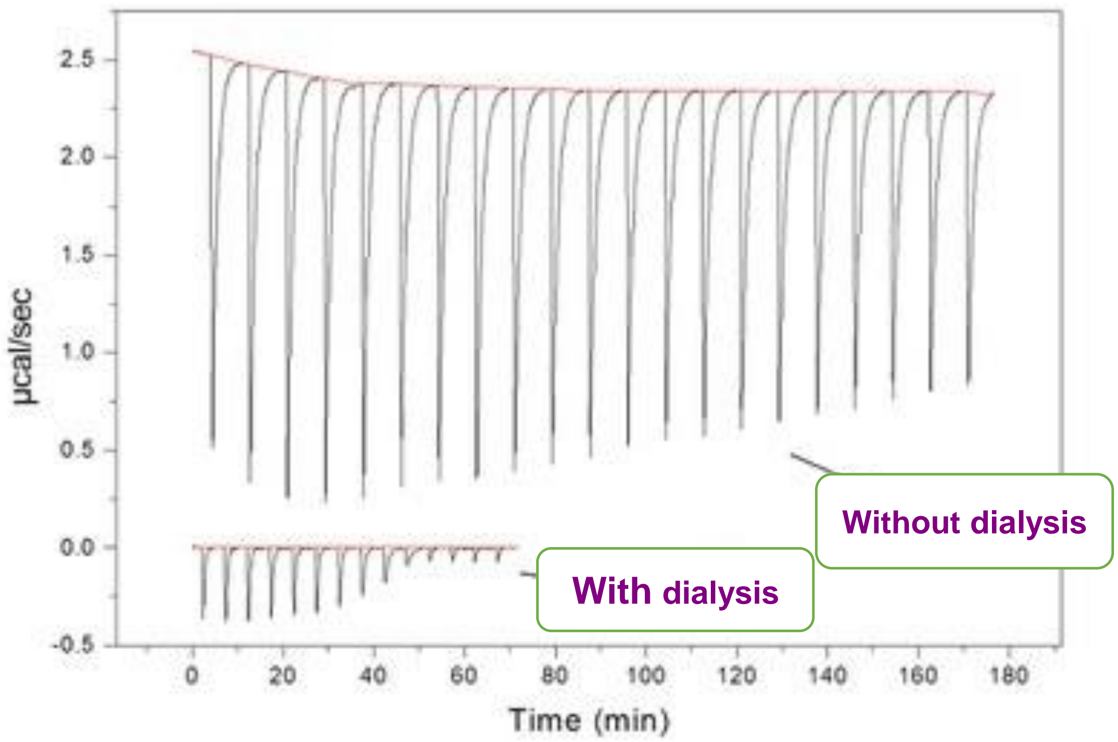
Sample preparation

- Check protein for aggregation (DLS, AUC)
- Ensure that protein and small molecule buffers are well matched!!!
  - **Dissolve** in same batch of buffer
  - **Dialyze** against same batch of buffer
  - Perform **buffer exchange** proteins
- Accurately determine sample concentration (at least A_{280} for protein)

Buffer (mis-)match

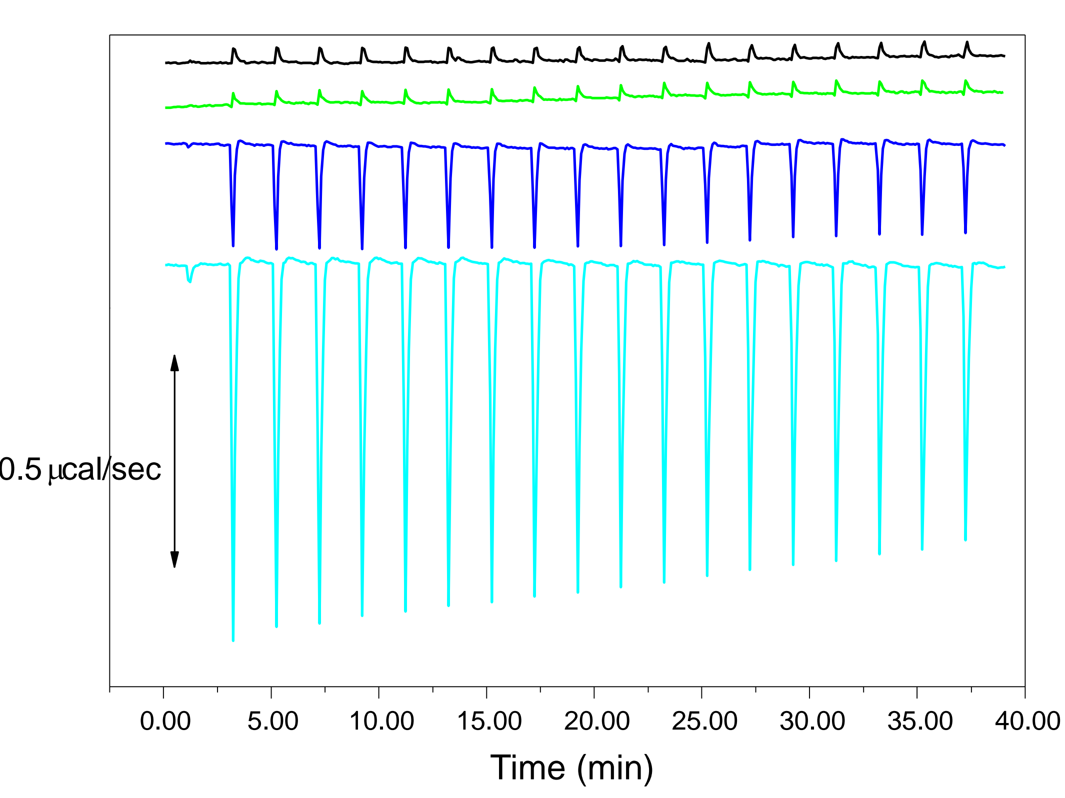
- Same sample **before** and **after** dialysis

- Large peaks due to differences in the NaCl concentration between buffers (heat of dilution)



DMSO in buffers

Large heats from DMSO dilution, if buffers are not matched



Buffer into buffer

5% DMSO into 5% DMSO

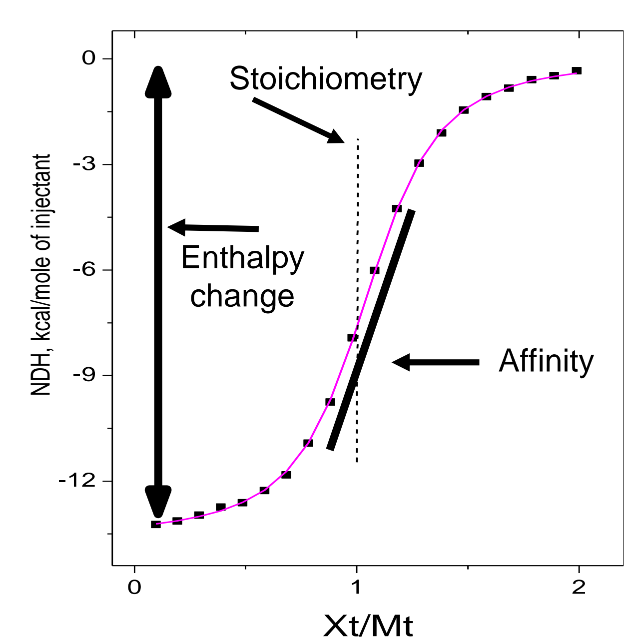
5% DMSO into 4.5% DMSO

5% DMSO into 4 % DMSO

Quality of the fit: fitted parameter *N* *number of binding sites*

$$Q = \frac{nM_t \Delta H V_o}{2}\left[1 + \frac{X_t}{nM_t} + \frac{1}{nKM_t} - \sqrt{\left(1 + \frac{X_t}{nM_t} + \frac{1}{nKM_t}\right)^2 - \frac{4X_t}{nM_t}}\right]$$

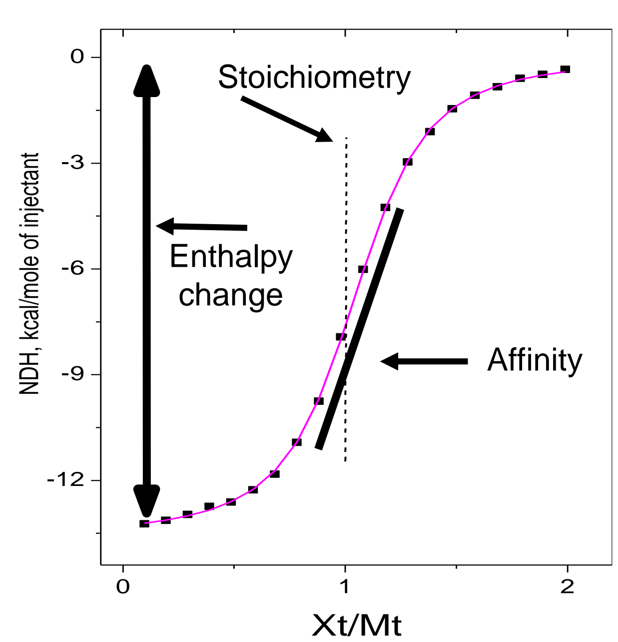
- "N" is the average number of binding sites per mole of protein in solution, assuming:
  - that all binding sites are identical and independent
  - that you have pure protein (and ligand)
  - that you have given the correct protein and ligand concentrations
  - that all your protein is correctly folded and active



Goodness of the fit: fitted parameter N
number of binding sites

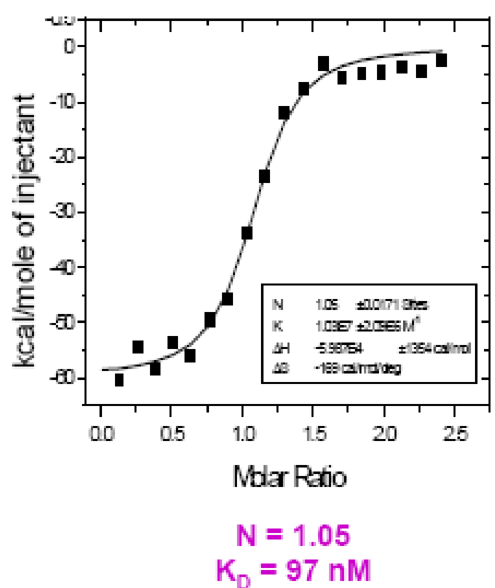
- If N≠ 1
- inaccurate input values for protein and/or ligand concentration
  - protein instability issues
  - compound solubility issues

- binding does not fit simple independent model
  - different number of binding sites
  - cooperativity/sequential binding



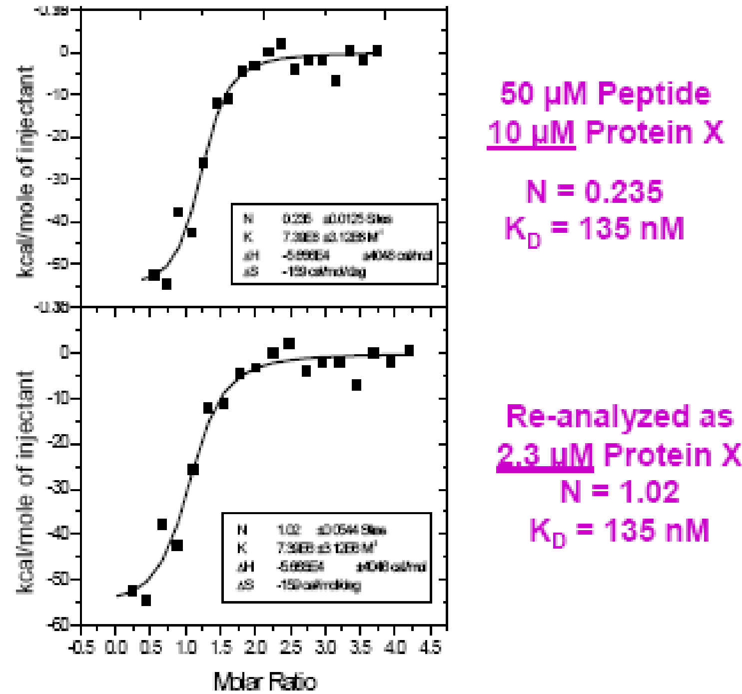
Assessment of protein quality by ITC

Peptide binding to protein Batch #1



N = 1.05
K_D = 97 nM

Peptide binding to protein Batch #2



50 µM Peptide
10 µM Protein X

N = 0.235
K_D = 135 nM

Re-analyzed as
2.3 µM Protein X
N = 1.02
K_D = 135 nM

- ***100%*** of Batch 1 protein active based on stoichiometry
- ***23%*** of Batch 2 protein active based on stoichiometry

Presented by L.Gao (Hoffmann-La Roche), poster at SBS 2009

Stoichiometry: Incorrect [Ligand]

n<1	n=1	n>1
N = 0.82	N = 1.02	N = 1.28
K_a = 6.89E4	K_a = 5.54E4	K_a = 4.43E4
ΔH = -1.69E4	ΔH = -1.36E4	ΔH = -1.09E4

Stoichiometry: Incorrect [Protein]

n<1

n=1

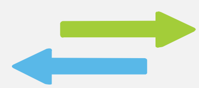
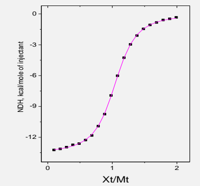
n>1

N = 0.82	N = 1.02	N = 1.28
K_a = 5.54E4	K_a = 5.54E4	K_a = 5.54E4
ΔH = -1.36E4	ΔH = -1.36E4	ΔH = -1.36E4

Inaccurate concentration effects

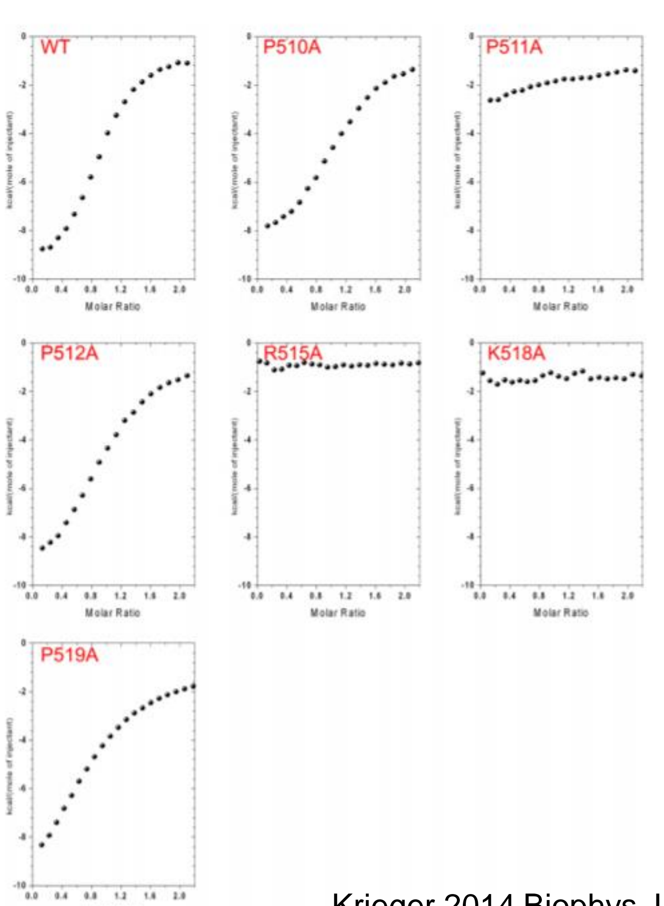
- **Error in syringe concentration** results in **error in DH, K and N !**
- **Error in cell concentration** results in **error in N**
- Put the sample of which you have most control over in the syringe and evaluate accordingly

Microcalorimetry in cube:

Microcalorimetry	Broad dynamic range	Information rich	Ease-of-use
• Direct measurement of heat change (ITC) • Direct measurement of melting transition temperature to predict thermal stability (DSC)	• Native molecules in solution (biological relevance) • Very sensitive to accomodate range of affinities	• All binding parameters (affinity, stoichiometry, enthalphy and entropy) in a single ITC experiment	• No labeling or immobilzation necessary • Wide range of solvent/buffer conditions
			

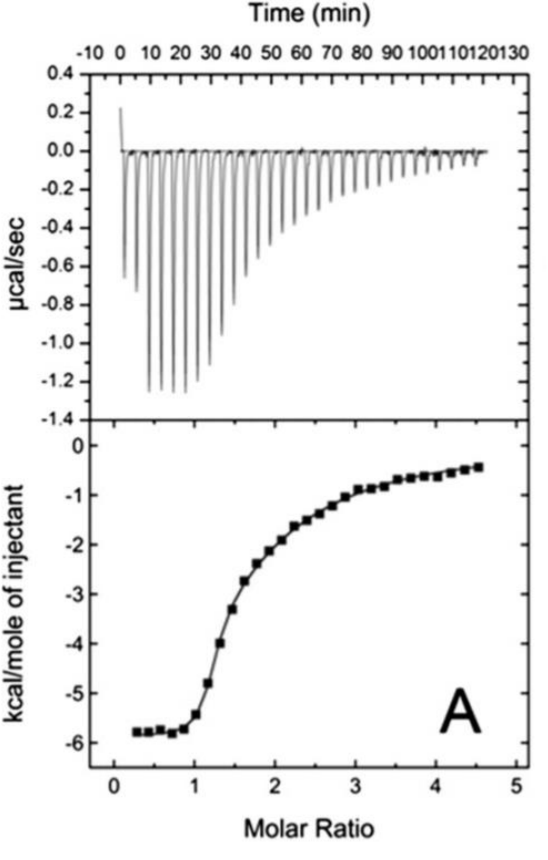
IDPs studied by calorimetry

Grb2 SH3 and Gab2$_{503-524}$ interaction



Krieger 2014 Biophys J

Myb32 interaction with KIX



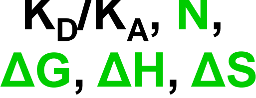
Arai 2015 PNAS

Isothermal titration calorimetry (ITC)

Advantages	Disadvantages
No labeling	Higher sample consumption
No immobilization	High concentration sample needed
Direct access to thermodynamics of interaction	Buffer matching is crucial
No limit in molecular size	Sample stirring (stability, viscous environment)

Comparison

	ITC	SPR	BLI	MST
Parameters	K_D/K_A, N, ΔG, ΔH, ΔS	K_D/K_A, k_a, k_d, (ΔG, ΔH, ΔS)	K_D/K_A, k_a, k_d	K_D/K_A, N, (ΔG, ΔH, ΔS)
K_D range	$10^{-12} - 10^{-2}$	$10^{-13} - 10^{-3}$	$10^{-11} - 10^{-3}$	$10^{-11} - 10^{-1}$
Sensitivity	Medium	High	Medium	High
Speed	30-120 min	15-120 min	30-120 min	15-30 min
No Labeling	✓	✓	✓	✗
No Immobilization	✓	✗	✗	✓
Sample consumption	Medium	Low	Low	Low
Complex samples	✗	✓	✓	✓
Real time	✓	✓	✓	✗
Automatization	✓	✓	✓	✓



Take home message

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

TRY SEVERAL APPROACHES !

Thank you for your attention

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