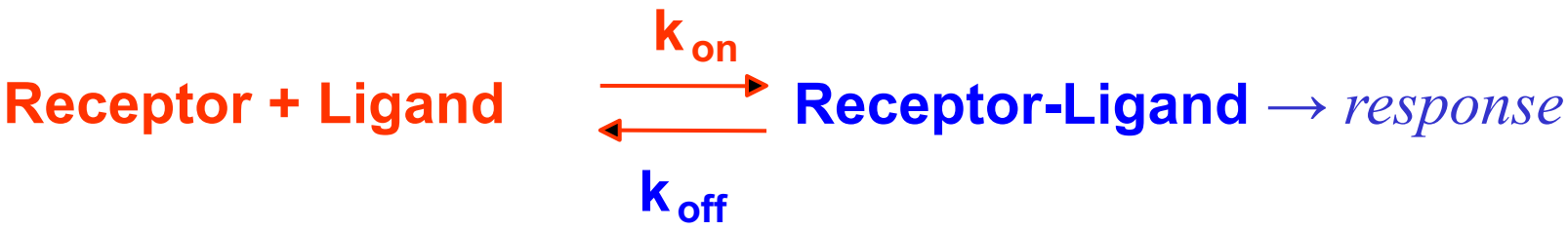
Quantitative analysis of protein-protein interactions

Binding assays

Receptor-ligand (protein-protein) binding equilibrium



k_{on} and k_{off} are the kinetic association and dissociation constants.

Association rate is given by

$$v_{on} = k_{on} \times [\text{Ligand}] \times [\text{Receptor}]$$

Dissociation rate of the complex is given by

$$v_{off} = k_{off} \times [\text{Receptor-Ligand}]$$

At equilibrium: $v_{on} = v_{off}$

$$k_{on}[R][L] = k_{off}[RL]$$

rearranging

$$\frac{[R][L]}{[RL]} = \frac{k_{off}}{k_{on}} = K_D$$

$$K_A = \frac{k_{on}}{k_{off}} = \frac{[RL]}{[R][L]}$$

when $[L] = K_D$ $\quad \frac{[R]}{[RL]} = 1$ $\quad [R] = [RL]$

K_{eq} (expressed as K_D or K_A) is the concentration of (free) ligand at which half of the binding sites are occupied.

Since $[R] = [R_{tot}] - [RL]$

$$K_D = \frac{[R][L]}{[RL]}$$

$$[RL] = \frac{[R]\,[L]}{K_D} = \frac{([R_{tot}] - [RL])\,[L]}{K_D}$$

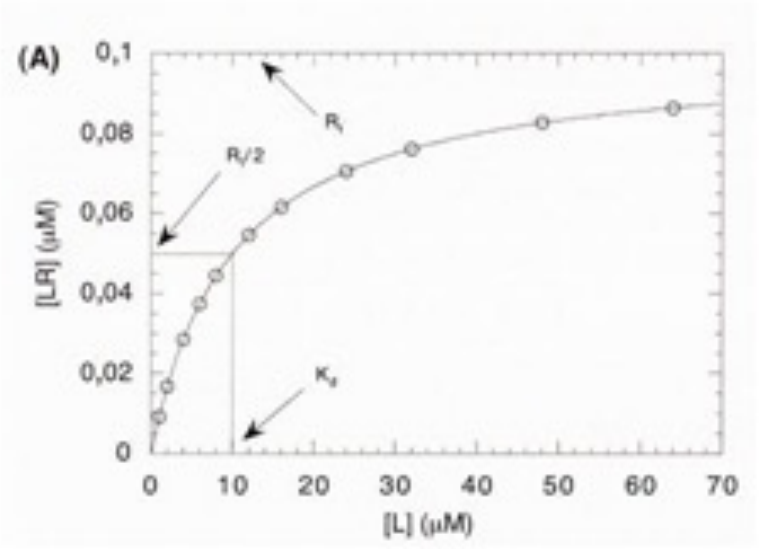
$$[RL]\,K_D = [R_{tot}]\,[L] - [RL]\,[L]$$

$$[RL]\,K_D + [RL]\,[L] = [R_{tot}]\,[L]$$

$$[RL]\,(K_D + [L]) = [R_{tot}]\,[L]$$

$$[RL] = \frac{[R_{tot}][L]}{K_D + [L]}$$

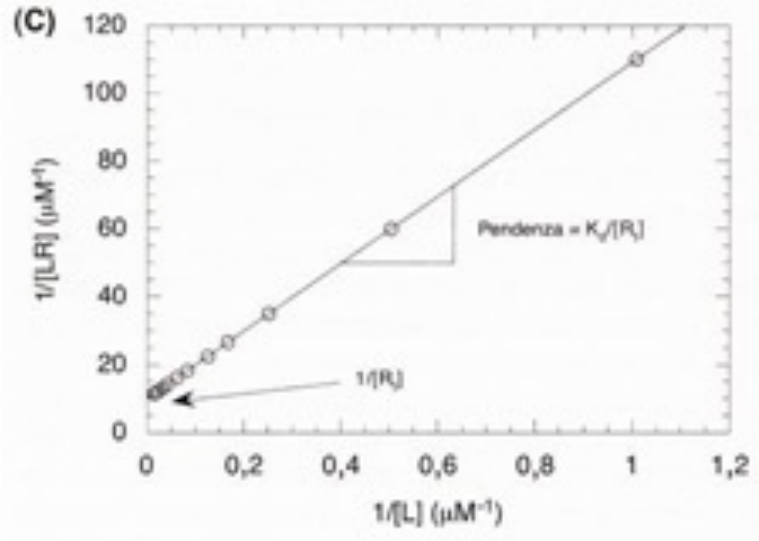
This equation allows to determine K_D by **measuring** [RL] at different ligand concentrations [L] when $[L] >> [R_{tot}]$, and thus $[L] \cong [L_{tot}]$



L'equazione

$$[LR] = [R_t]\frac{[L]}{[L] + K_d}$$

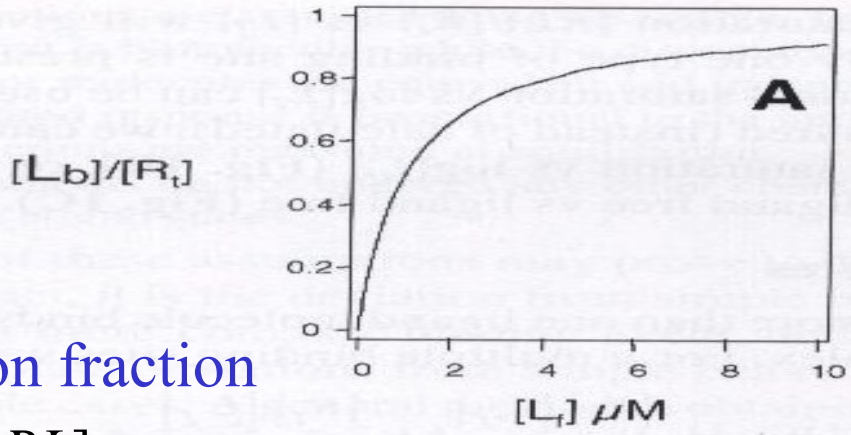
è quella di una iperbole rettangolare



La trasformazione lineare della stessa equazione (grafico dei doppi reciproci)

$$\frac{1}{[LR]} = \frac{K_d}{[R_t]}\left(\frac{1}{[L]}\right) + \frac{1}{[R_t]}$$

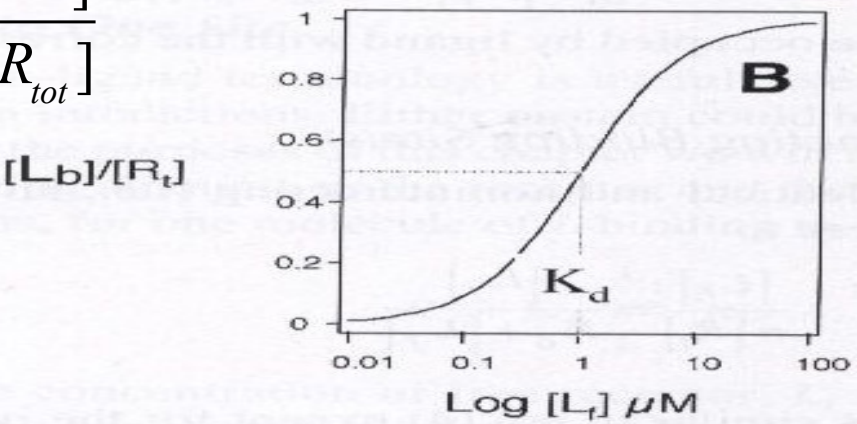
consente di calcolare graficamente la K_d



Rectangular hyperbola

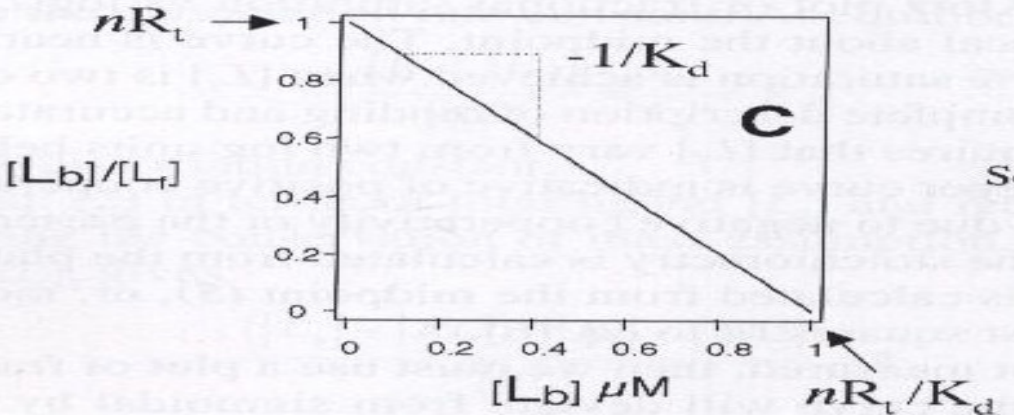
Saturation fraction

$$Y = \frac{[RL]}{[R_{tot}]}$$



Klotz plot

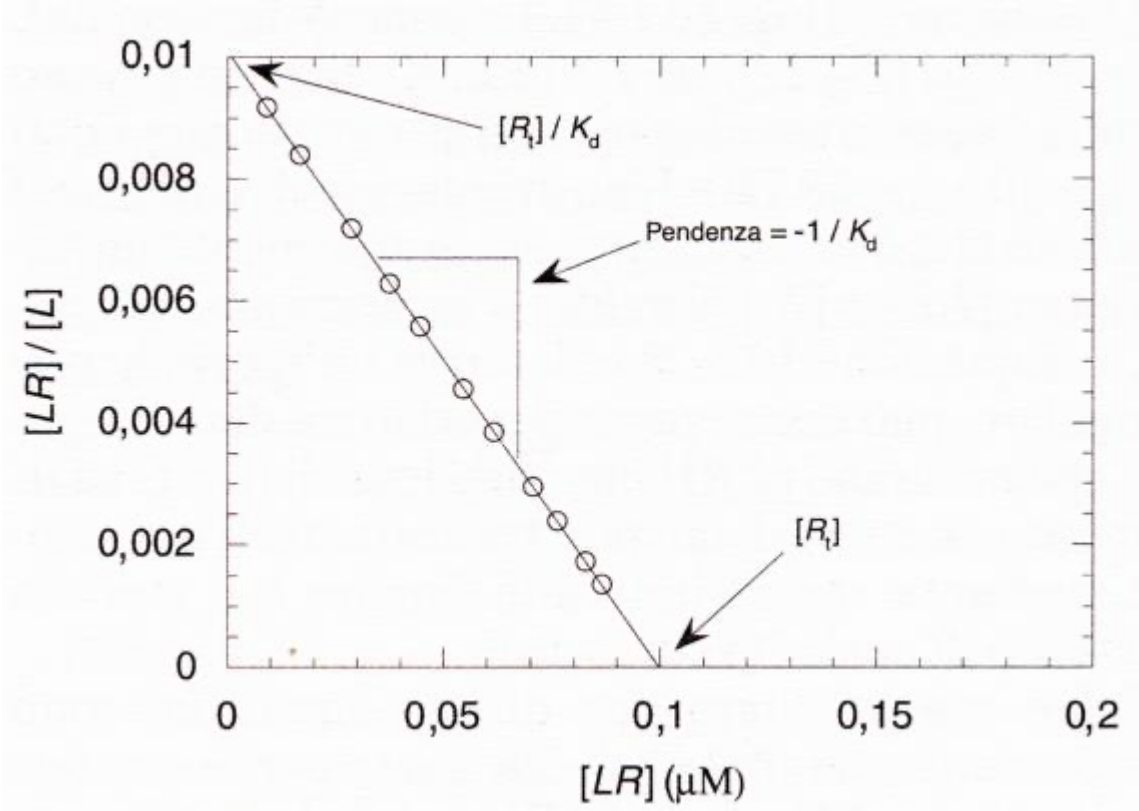
semilog



Scatchard plot

Another linear transformation that was frequently used is the Schatchard equation

$$\frac{[RL]}{[L]} = \frac{[R_{tot}]}{K_d} - \frac{[RL]}{K_d}$$



It allows a graphic estimation of K_d

Scatchard analysis

Starting from equation:

$$[RL] = \frac{[R_{tot}]\ [L]}{K_d + [L]}$$

$$[RL]\ ([L] + K_d) = [R_{tot}]\ [L]$$

$$[RL]\ [L] + [RL]\ K_d = [R_{tot}]\ [L]$$

Dividing by $[L]\ K_d$:

$$\frac{[RL]\ [L]}{[L]\ K_d} + \frac{[RL]K_d}{[L]\ K_d} = \frac{[R_{tot}]\ [L]}{[L]\ K_d}$$

then: $$\frac{[RL]}{[L]} = \frac{[R_{tot}]}{K_d} - \frac{[RL]}{K_d}$$

If **B** (Bound) is the concentration of bound ligand [RL];

n $[R_{tot}]$ is the number of binding sites

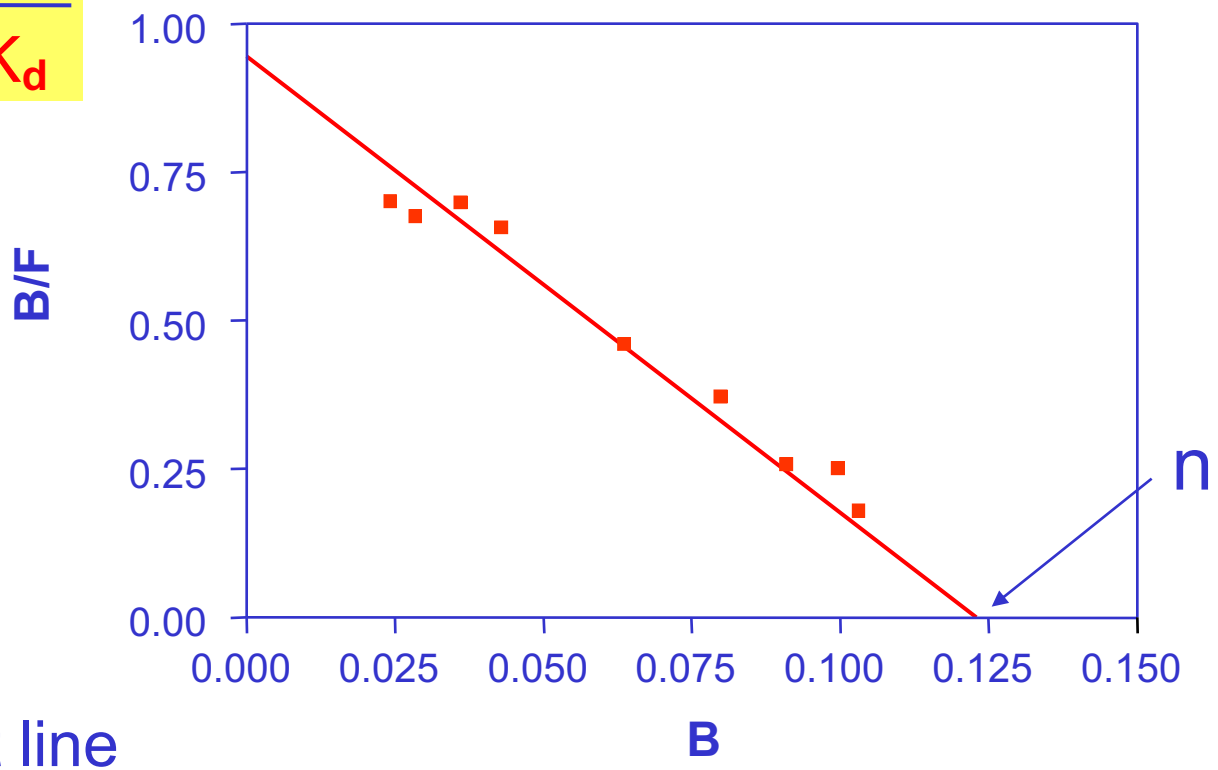
F (Free) is the concentration of free ligand [L]

we will have:

$$\frac{B}{F} = -\frac{B}{K_d} + \frac{n}{K_d}$$

The graph of the ratio of bound/free ligand **(B/F)** versus concentration of bound ligand **(B)** will be:

$$\frac{B}{F} = -\frac{B}{K_d} + \frac{n}{K_d}$$



We obtain a straight line

$$\text{slope} = -\frac{1}{K_d}$$

The abscissa intercept **(B/F = 0)** allows to determine **n** that represents the number of binding sites.

Effect of L and R concentration

- To obtain reliable results it is important to measure [RL] when [L] is close to K_D
- Values are accurate when $[R_{tot}] << [L_{tot}]$ and $< K_D$, so $[L_{tot}]$ is almost equal to [L]
- The binding assay should be sufficiently sensitive to allow to employ concentrations of L and/or R between 0.1 and 10 K_D

Effect of L and R concentration

If the binding assay is carried out in conditions where [R] = [L] it is necessary to substitute in equation

$$[RL] = \frac{[R_{tot}][L]}{K_D + [L]}$$

$[L] = [L_{tot}] - [RL]$

$$[RL] = \frac{[R_{tot}]([L_{tot}] - [RL])}{K_D + ([L_{tot}] - [RL])}$$

Rearranging it becomes

$$K_D\,[RL] + [L_{tot}]\,[RL] - [RL]^2 = [R_{tot}]\,[L_{tot}] - [R_{tot}]\,[RL]$$

Effect of L and R concentration

$$[RL]^2 - (K_d + [R_{tot}] + [L_{tot}])\,[RL] + [R_{tot}]\,[L_{tot}] = 0$$

This is a quadratic equation

$$ax^2 + bx + c = 0$$

that can be solved with the formula

$$x = \frac{-b \pm \sqrt{b^2 - 4ac}}{2a}$$

$$[RL] = \frac{(K_D + [R_{tot}] + [L_{tot}]) - \sqrt{(K_D + [R_{tot}] + [L_{tot}])^2 - 4[R_{tot}][L_{tot}]}}{2}$$

TECHNIQUES FOR THE QUANTITATIVE STUDY OF RECEPTOR-LIGAND BINDING

The capacity to quantitatively analyze a binding equilibrium depends on the possibility to distinguish between free and bound receptor (or free and bound ligand) and measure at least one among L, R and RL

Ideally the concentration of one of the two components is fixed at a value $<<K_D$ and the concentration of the other component is varied in an interval between 0.1 K_D and 10 K_D

Binding studies

Saturation analysis: Study of saturation of the receptor at equilibrium at different concentrations of ligand

Kinetic analysis: Study of the kinetics of association and dissociation of the ligand and receptor as a function of concentration

Methods without physical separation of free and bound ligand

Binding of the ligand to the receptor must change a measurable property of L and/or R

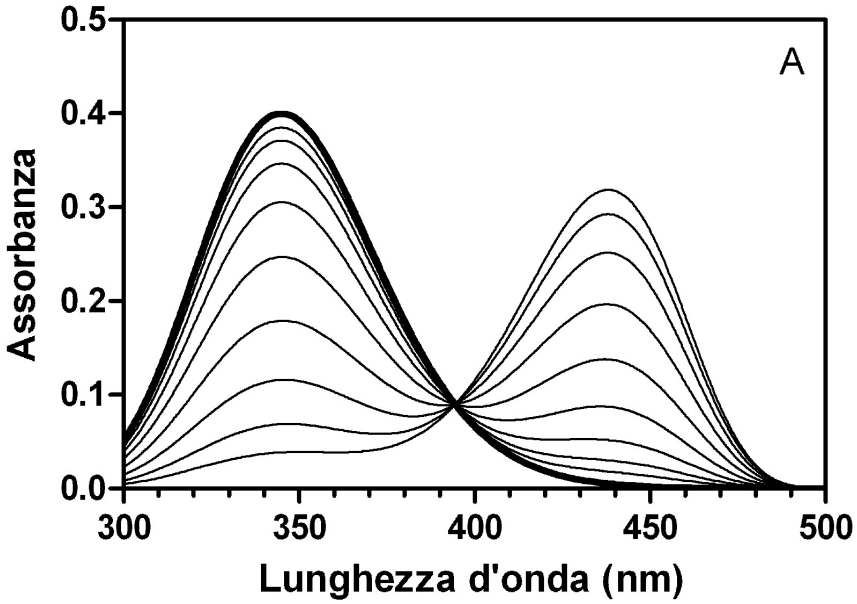
- Absorption spectroscopy (alteration of extinction coefficient, change of wavelength of absorption peaks)
- Fluorescence spectroscopy (alteration of intensity or wavelength of emission)
  - Intrinsic fluorescence (tryptophan and tyrosine)
  - Labelling with fluorescent probes (fluorophores)

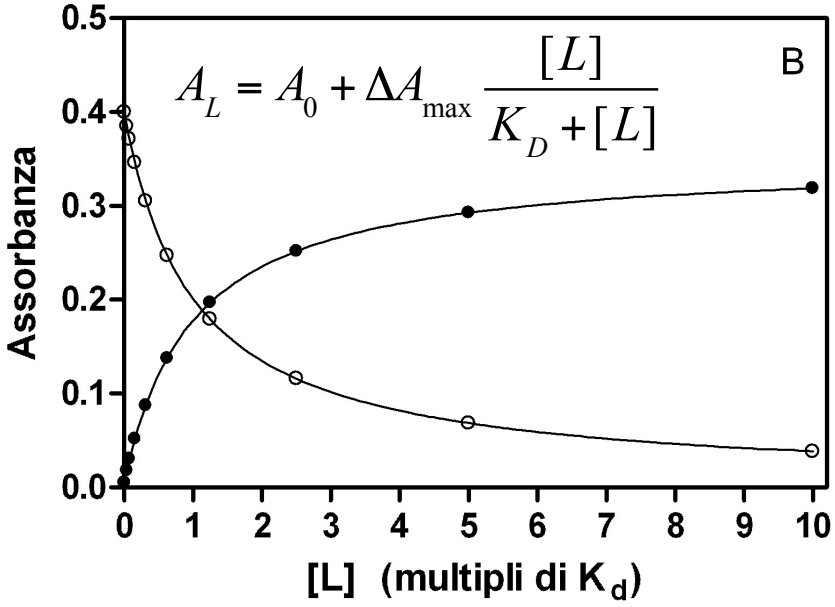
- ITC: Isothermal titration Calorimetry (measure of binding enthalpy)
- SPR: Surface Plasmon Resonance (measure of the change of refractive index)

Binding of the ligand to the receptor must change a measurable property of L and/or R ➔ if the concentration of complex RL is proportional to the measured 'signal' change then:

$$[RL] = \frac{[R_{TOT}][L]}{K_D + [L]}$$

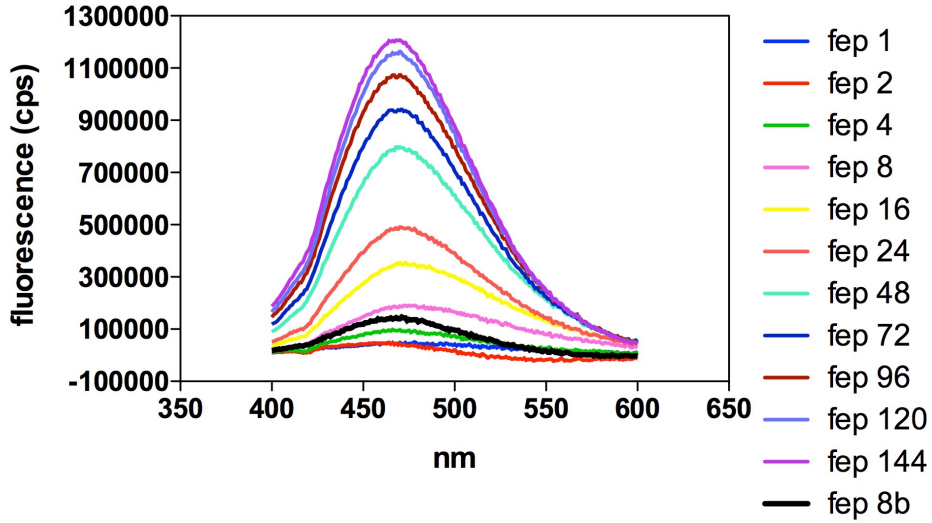
$$\Delta S = \frac{\Delta S_{max}\,[L]}{K_D + [L]}$$

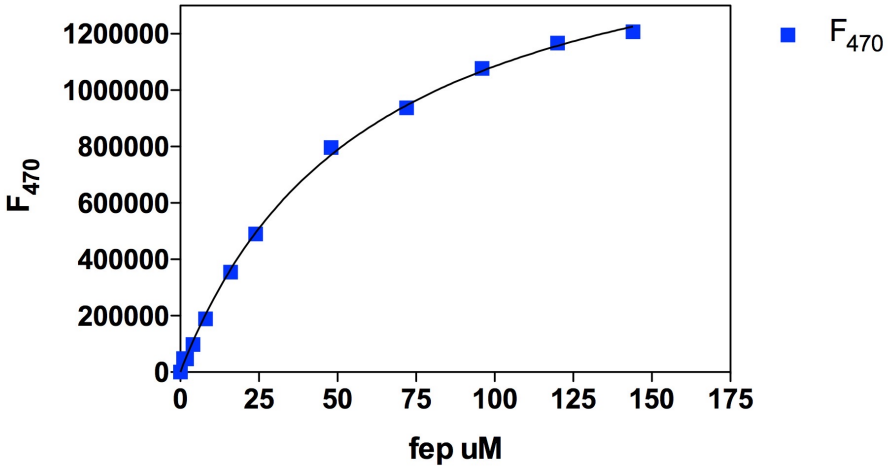




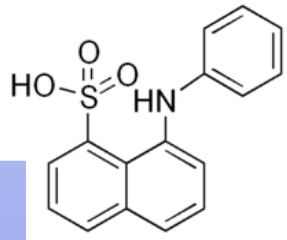
Spectral variations observed by addition of increasing concentrations of L at a fixed concentration of R. Panel A. The absorption spectrum in bold is R in the absence of ligand. The other spectra are obtained by addition of increasing concentrations of L. As L increases, a decrease of the protein absorption band at 350 nm due to the presence of a cofactor, is observed. A corresponding increase of a band with a maximum at 440 nm, due to formation of the RL complex can be seen. Panel B. The graph shows the variations of absorbance at 350 nm (open symbols) and at 440 nm (closed symbols) measured as a function of the concentration of L.

Analysis of Fep1-ANS binding





Fluorescence spectra (λ_{exc} 370 nm)
Titration of ANS 10 μM with Fep1 1-144 μM
ANS fluorescence increases when the probe is bound to proteins



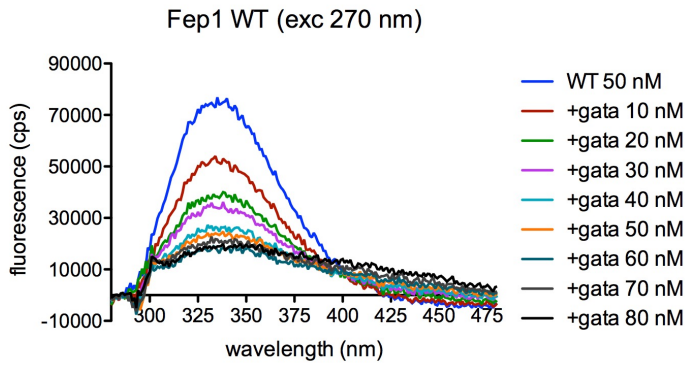
$$\Delta S = \frac{\Delta S_{max}\,[L]}{K_D + [L]}$$

K_D 60 μM
ΔS_{max} $1.74 \cdot 10^6$ cps

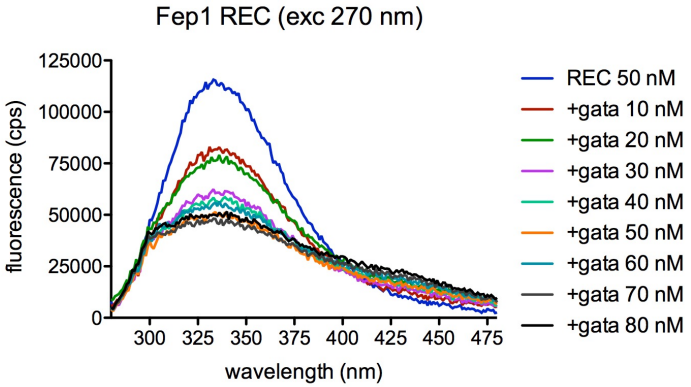
Analysis of Fep1-DNA binding

- Intrinsic fluorescence spectra of tyrosine and tryptophan (λ_{exc} 270 nm and 295 nm)
- Titration of Fep1 50 nM with dsDNA GATA 10-80 nM
- Fep1 fluorescence decreases when it binds DNA
- K_D about 30 nM

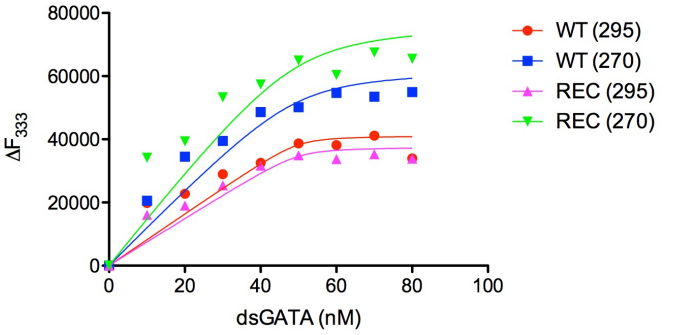
A



B



C



Isothermal Titration Calorimetry (ITC)

ITC measures heat absorbed or released in a binding reaction in a sequential way (by titration)

Label-free technique in solution

Parameters that are determined are K_A, n, ΔH, ΔS and ΔG

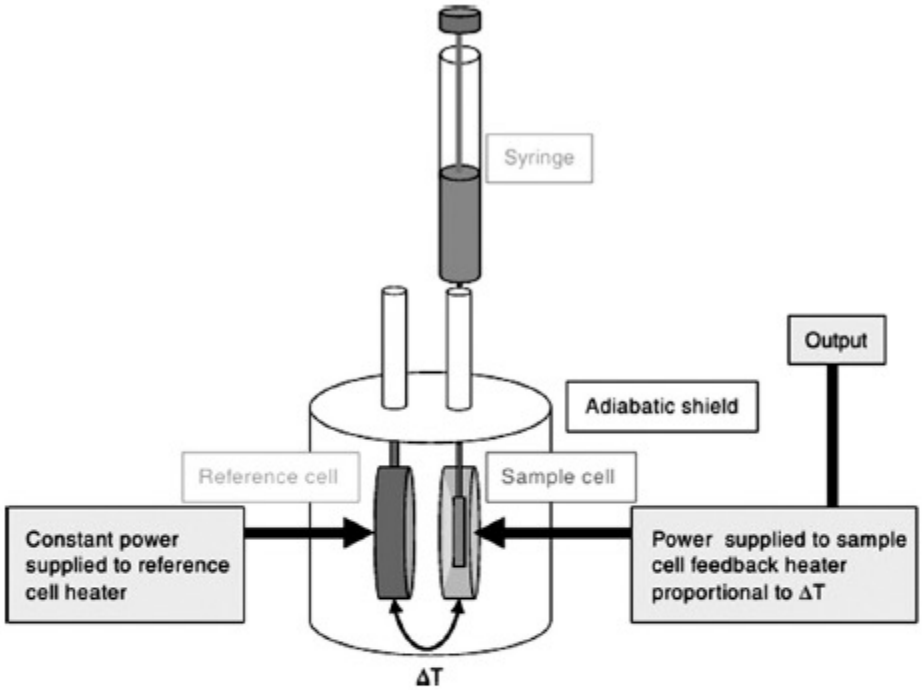


Fig. 1. Illustration of a standard power compensating isothermal titration calorimeter. Two chambers inside are surrounded by adiabatic shield, one is used as a reference cell and the other as reaction cell. A thermoelectric device measures the temperature difference between the two cells. After each injection of a titrant into sample cell filled with an analyte, the two materials interact, and certain amount of heat is generated or absorbed. The temperature difference between the sample and reference cells (ΔT) is kept at a constant value (i.e. baseline) by the addition or removal of heat to the sample cell. The integral of the power required to maintain $\Delta T = 0$ over time is a measure of total heat resulting from the process being studied. To keep the solution homogeneous after addition of titrant spinning syringe with a paddle shape needle tip is used for injecting and subsequent mixing of the reactants [15].

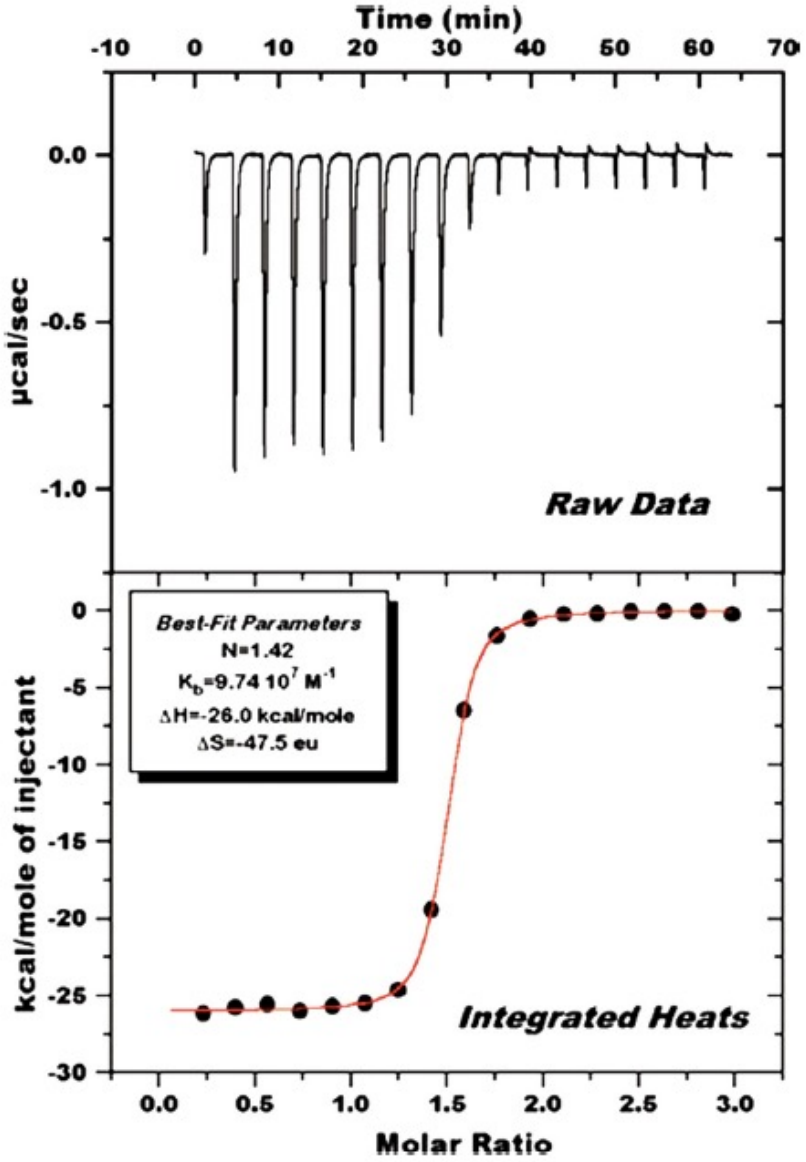


Fig. 2. Representative ITC measurement. Eighteen injections of ligand solution are added to protein solution in the ITC cell. The area of each injection peak (top panel) is equal to the total heat released from that injection. When this integrated heat is plotted against the molar ratio of ligand added to macromolecule in the cell, a complete binding isotherm for the interaction is obtained (bottom panel). The one site model was used to fit the data. The solid red line is the calculated curve using the best-fit parameters. The values describing stoichiometry, binding constant, and enthalpy are shown in the box [28].

Isothermal Titration Calorimetry (ITC)

- $K_D = \exp(-\Delta G/RT)$ $\Delta G = -RT\ln K$
- $\Delta G = \Delta H - T\Delta S$
- Protein concentration should be between 10 and 500 times K_D
- $c = n\, M_T/K_D$
- The heat associated with each injection is

$$q_i = v\, \Delta H\, \Delta L_{Bi}$$

- For binding to *n* independent sites

$$\frac{dL_B}{dL_T} = \frac{1}{2} + \frac{1 - \Phi - 1/c}{2 \times ((1 + \Phi + 1/c)^2 - 4\Phi)^{1/2}}$$

where $\Phi = L_T/nM_T$

M_T total protein concentration

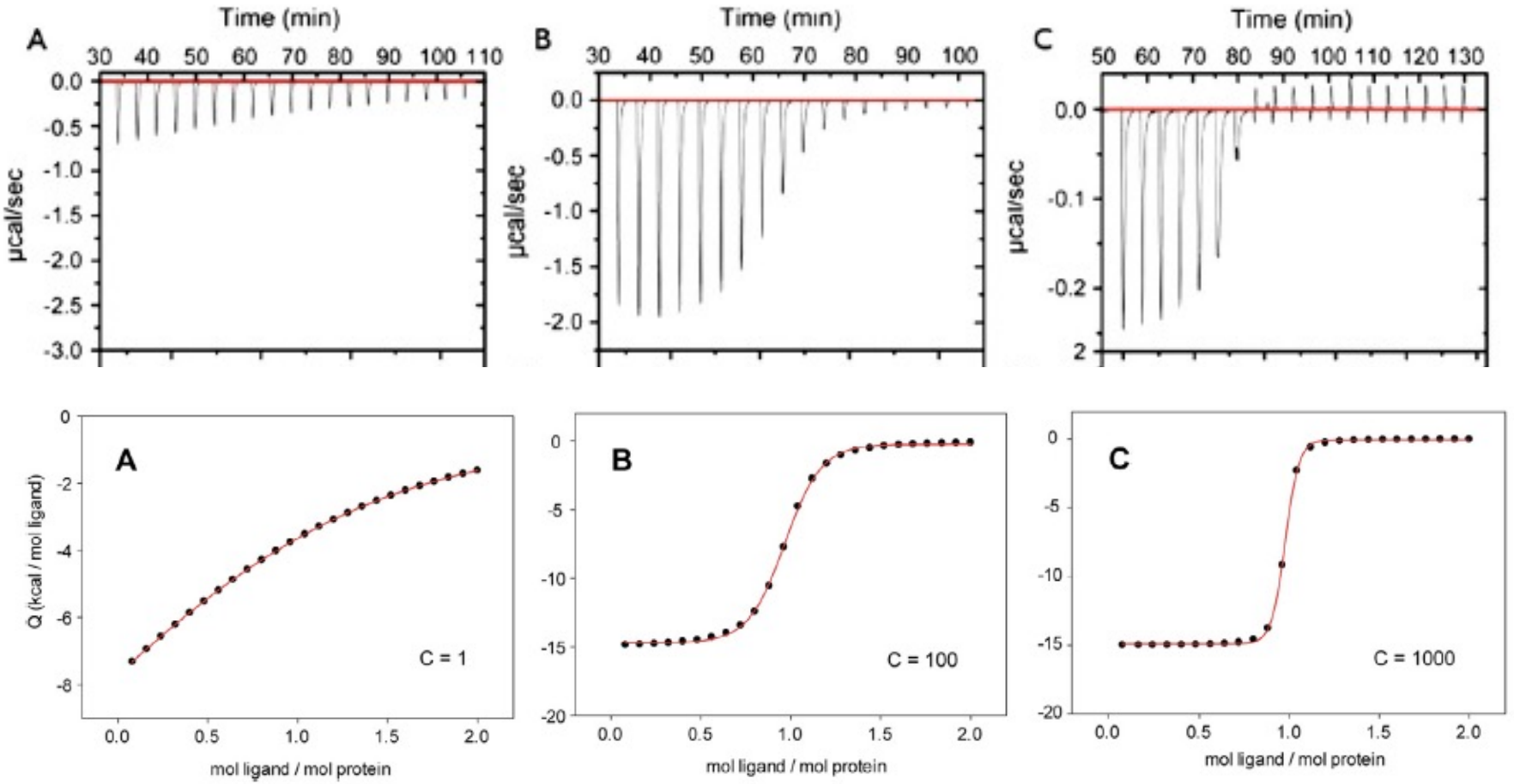


Fig. 1. Binding isotherms for low (A) intermediate (B) and high (C) affinities. Integrated injection heats produce binding isotherms, from which K_D, ΔH and n are determined. Simulated data are shown with ΔH of −15 kcal/mol, n of 1. The solid red lines through the data are fits to the independent binding sites model. Corresponding c values are on the panels.

Zinc fingers as protein recognition motifs: Structural basis for the GATA-1/Friend of GATA interaction

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GATA-1 and Friend of GATA (FOG-1) are two zinc-finger transcription factors essential for correct erythropoiesis.

Analysis of DNA - GATA-1 - FOG-1 interaction: pull-down on DNA immobilized on magnetic beads of nuclear extracts (A) or purified GATA-1 ZnF1-MBP and GST-FOG-11 ZnF6 (B)

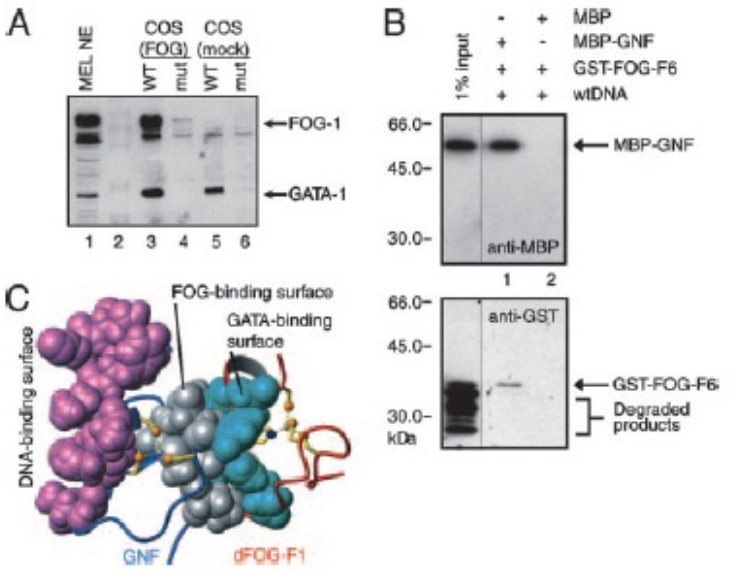
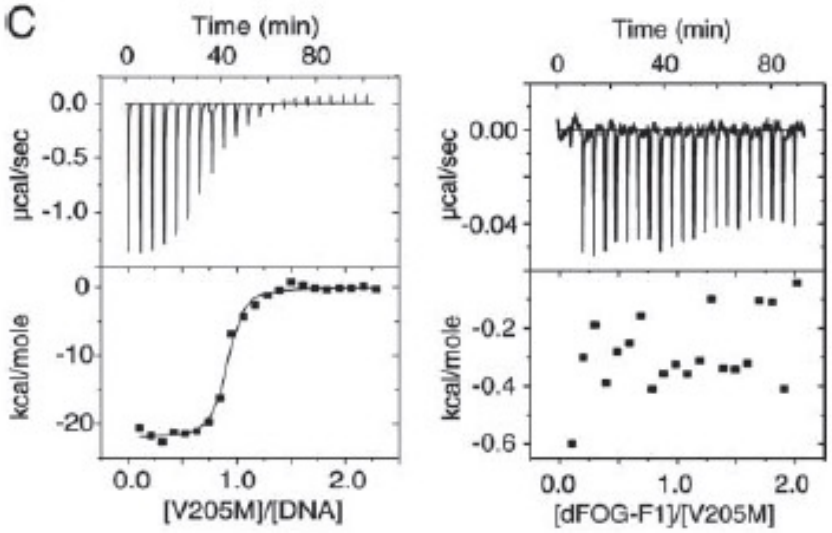


Fig. 4. Simultaneous binding of GATA-1 to DNA and FOG-1. (*A*) Pull-down showing that full-length GATA-1 can simultaneously bind to the murine α-globin promoter and recruit full-length FOG-1. MEL cell nuclear extract (lane 1), supplemented with COS nuclear extract from untransfected [COS-(mock); lanes 5 and 6] or transfected [COS(FOG); lanes 3 and 4] cells, was incubated with DNA containing either a wild-type (WT) or a mutant (mut) GATA site coupled to magnetic beads. Complexes were blotted with antibodies against GATA-1 and FOG. (*B*) Pull-down showing that murine GATA-1 NF can simultaneously bind a GATC site and FOG-1 finger 6. Magnetic beads coupled to a GATC-containing oligonucleotide were incubated with purified MBP-NF and GST-FOG-F6 and run on denaturing gels. Gels were blotted with anti-MBP and anti-GST. A number of breakdown products of GST-FOG-F6 are visible in the input. (*C*) DNA- and protein-binding surfaces of the GATA/FOG complex. The DNA-binding surface of GATA-1 NF is inferred from the NMR structure of the GATA-1 CF bound to DNA (38). The complex is shown in the same orientation as in Fig. 1*D*.

Effect of mutations on GATA-1 that cause anemia: V205M, G208S, R216Q.
Analysis of GATA-DNA and GATA-FOG binding



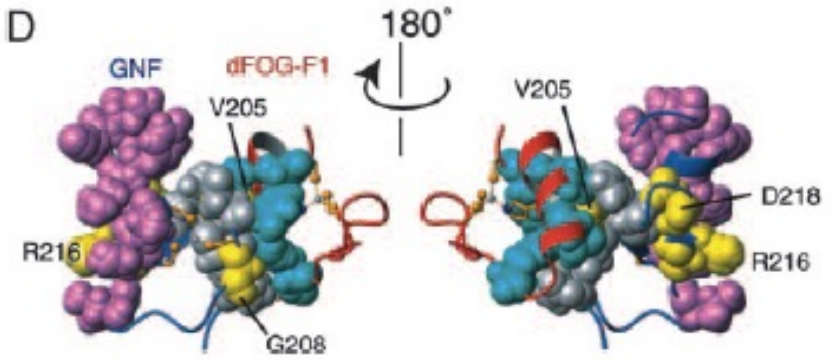


Table 1. DNA- and FOG-binding affinities of naturally occurring GATA-1 mutants

	k_a	
Protein	NF:DNA, M^{-1}	NF:FOG, M^{-1}
mNF	$(1.9 \pm 0.1) \times 10^6$	$(4.5 \pm 1.8) \times 10^4$
V205M	$(2.0 \pm 1.6) \times 10^7$	No binding
G208S	$(9.1 \pm 5.2) \times 10^6$	No binding
R216Q	No binding	$(9.4 \pm 0.3) \times 10^4$
D218G*	$>10^7$	$(1.9 \pm 0.3) \times 10^4$

Association constants were measured by isothermal titration caliometry. Numbers are presented with standard deviations obtained from fitting the data to a simple 1:1 model.

*The sharpness of the binding curve obtained in the D218G/DNA titration prevented accurate determination of the binding affinity, and the affinity is therefore presented as a lower limit.

In some cases, these techniques can not be used and a direct measure of binding is impossible.

 it is necessary to employ techniques where, after reaching equilibrium, it is possible to measure **separately** at least one among L, R and RL.

Generally, radioactively labelled ligands with high specific activity are used to provide sensitivity

- ^{125}I 2200 Ci/mmole
- ^{3}H 2-20 Ci/mmole
- ^{14}C 0.05-0.5 Ci/mmole

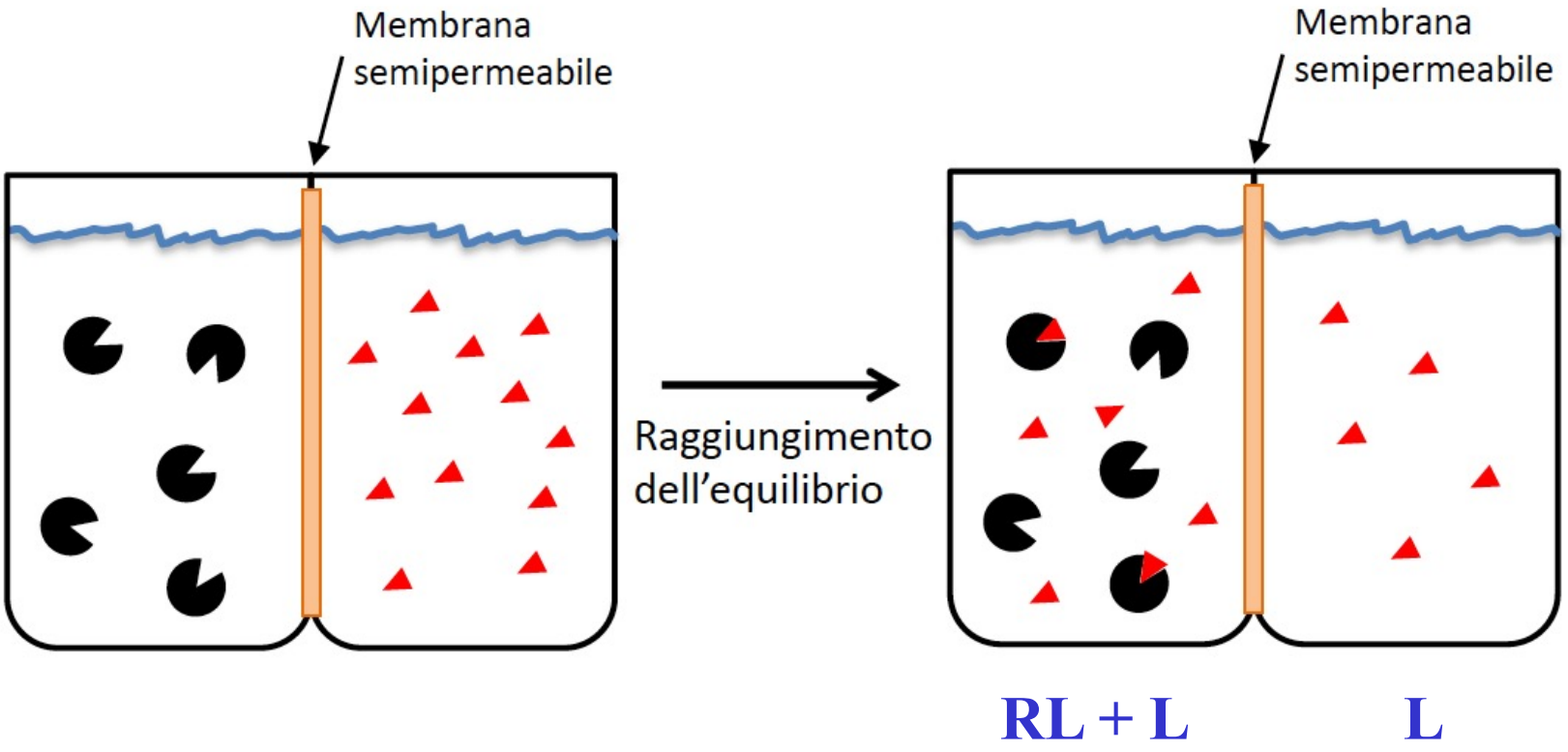
Radioactivity is measured in Bequerel (Bq, disintegrations per second) or in Curie (Ci, disintegrations of 1 gr of radium per second). 1 Ci = $3.7\ 10^{10}$ Bq

Methods with separation of free ligand (L) from ligand bound to the receptor (RL)

Centrifugation
Equilibrium dialysis
Ultrafiltration
Adsorption
Precipitation

Immunoprecipitation
Affinity chromatography

Equilibrium dialysis



Method	Measured quantity	Advantages	Disadvantages
Filtration and ultrafiltration	RL, L_{free}	Rapid, many replicas	Can not be used if $t_{1/2}$ of the complex is less than 15 sec
Equilibrium dialysis	RL + L_{free}, L_{free}	Equilibrium method	Slow
Gel-filtration	RL	Adequate for soluble receptors	Slow
Precipitation with PEG or TCA	RL, L_{free}	Adequate for soluble receptors, separation can be obtained by filtration or centrifugation	Can not be used if R and L are proteins and both precipitate with PEG or TCA
Centrifugation	RL, L_{free}	Adequate for membrane receptors	Slow, requires high speed centrifuge

These separation methods are effective if there is a large difference in the molecular weight of receptor and ligand

Experimental approach to saturation analysis with radioactive labelling

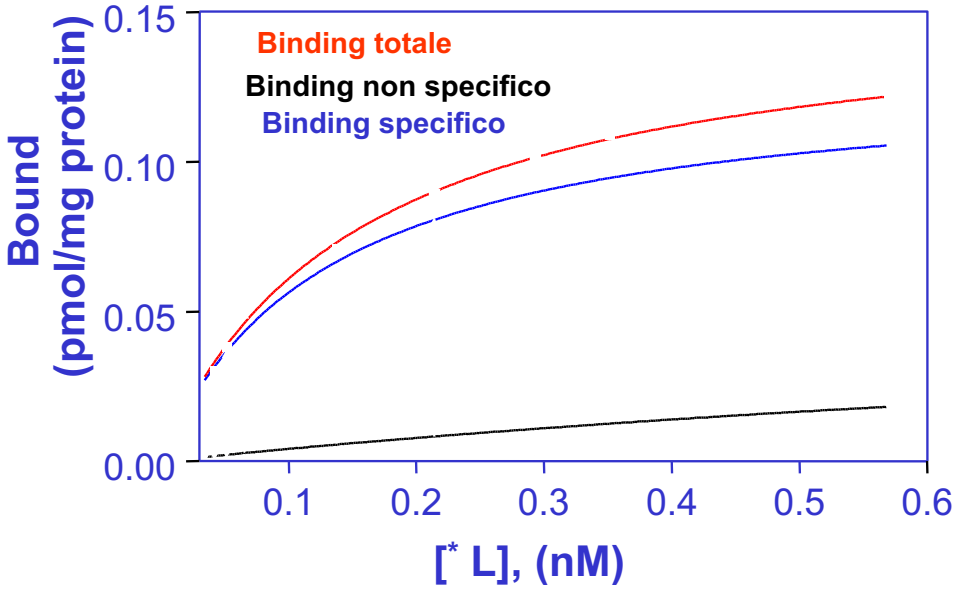
Total Binding and **Non-Specific Binding** are determined at different concentrations of (radiolabelled) ligand.

Total Binding

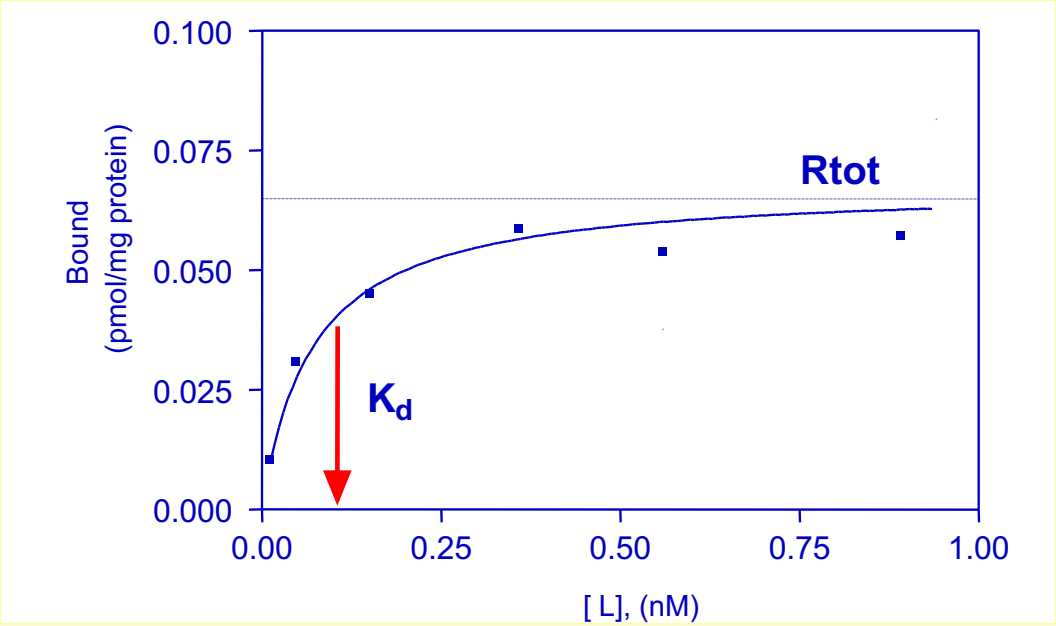
It is obtained by adding increasing amounts of radioligand to a fixed amount of receptor

Non-Specific Binding

It is obtained by adding a large excess of unlabelled ligand to displace any radioligand from the receptor, or by performing the assay in the absence of receptor



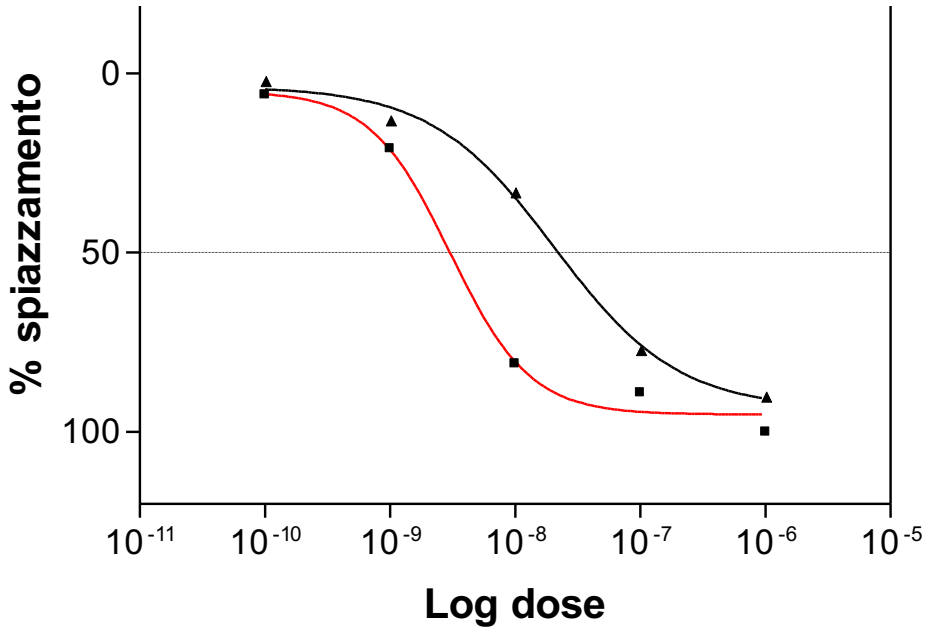
Visualizing only the specific binding curve it is possible to determine **K_d** and **Rtot**



Competition analysis

Competition binding is performed to determine the capacity of a ligand L2 to compete with a ligand L1 for a given receptor.

- Fixed concentrations of **labelled ligand L1** and receptor are used.
- The system is brought to equilibrium and the concentration of L1R complex in the presence of different concentrations of unlabelled L2 is measured.

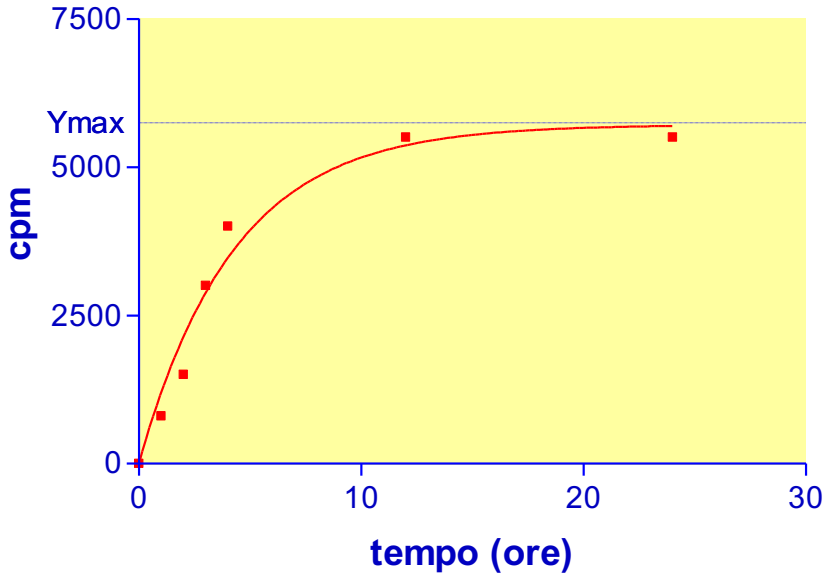


The IC_{50} value indicates the concentration of ligand L2 that displaces 50% of radioligand L1 from the receptor at equilibrium.

Kinetic analysis

Association kinetics

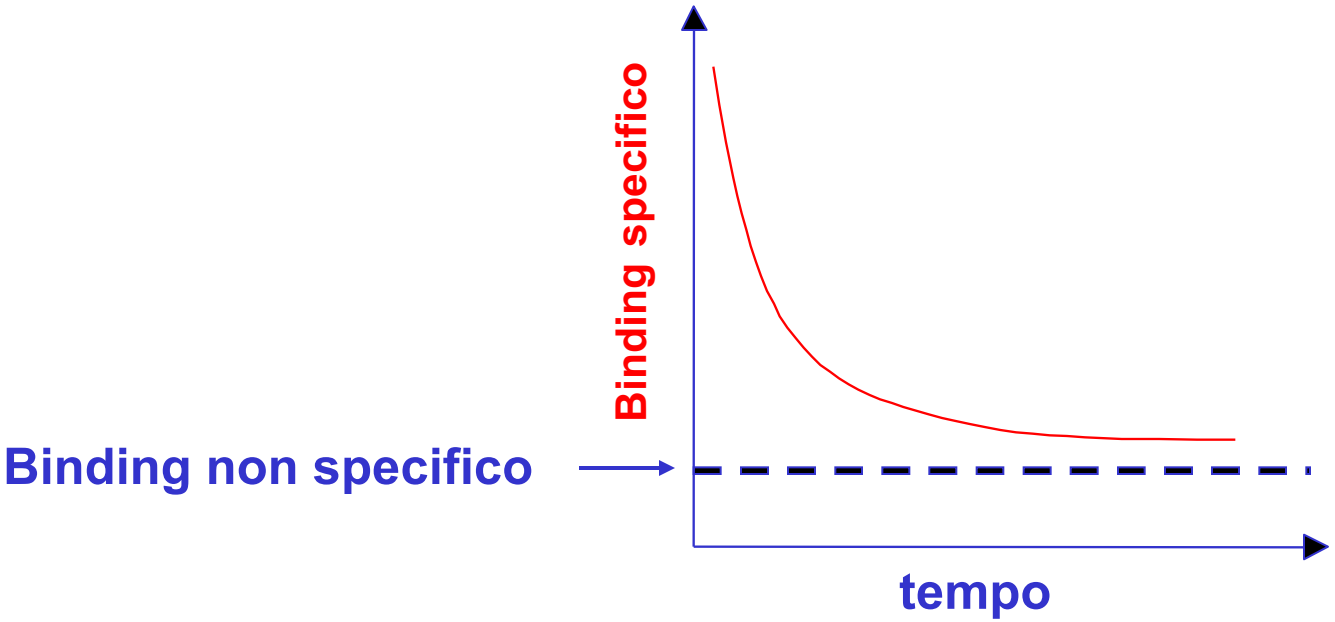
The experiment is performed to determine the association rate constant k_{on}.



The amount of RL complex increases with time up to a maximum equal to the specific binding at equilibrium (Y_{max}) for that concentration of ligand.

Association rate constant k_{on} (min^{-1} M^{-1}).

Dissociation kinetics

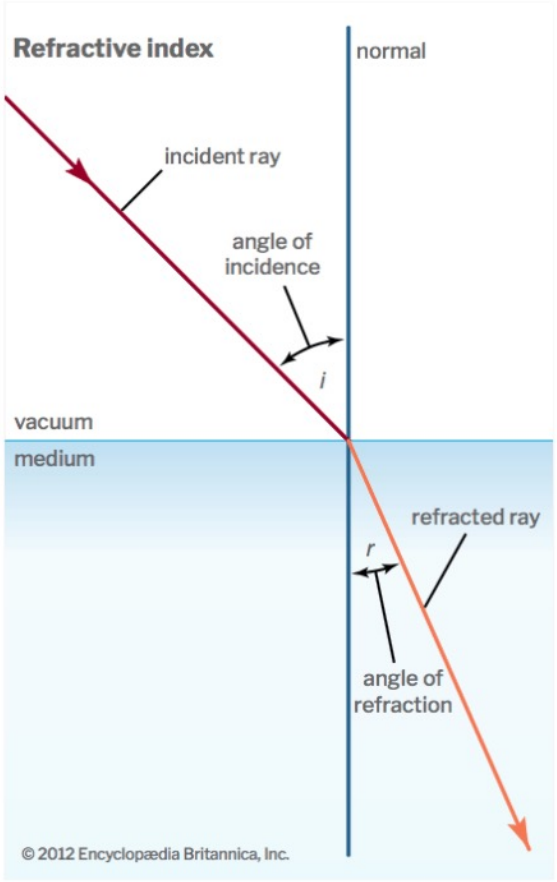


The receptor-radioligand system is brought at equilibrium and then excess cold ligand is added and the concentration of radiolabelled complex is measured over time.

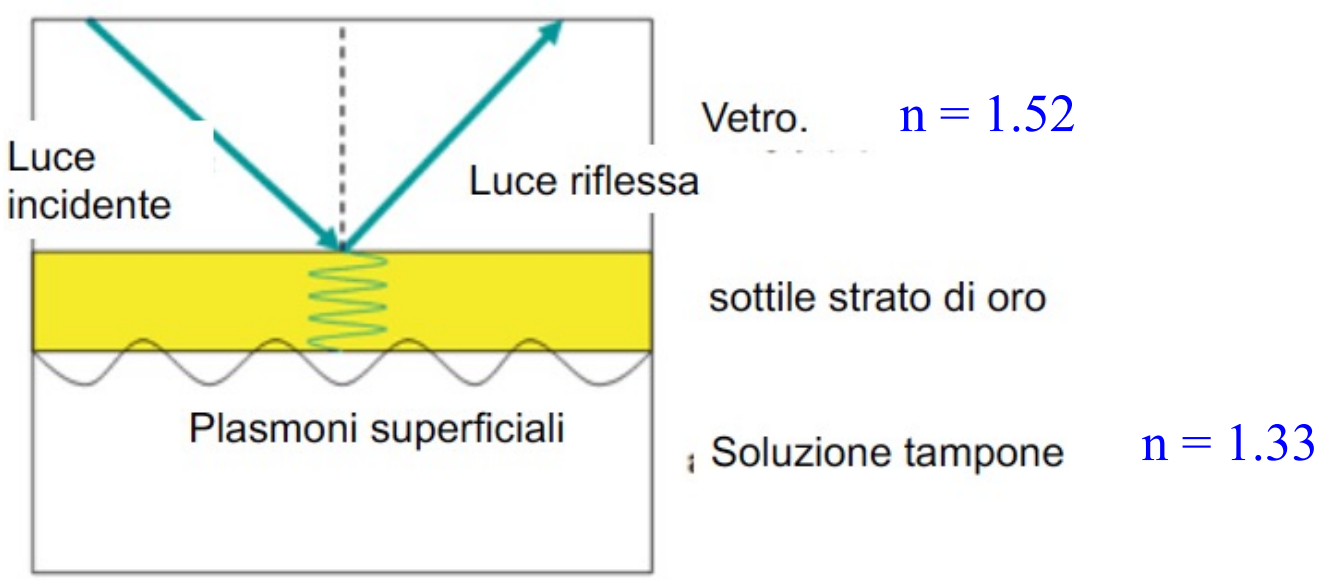
Dissociation rate constant k_{off} (min^{-1}).

SPR: surface plasmon resonance kinetic analysis of binding

- Technique used to measure molecular interactions in real time
- It is based on changes in refractive index (n) on the surface of a sensor (chip) caused by binding of an analyte to a ligand immobilized on the chip



SPR (surface plasmon resonance) is a physical process that is observed when a plane-polarized beam of light hits a metallic film in conditions of total internal reflection

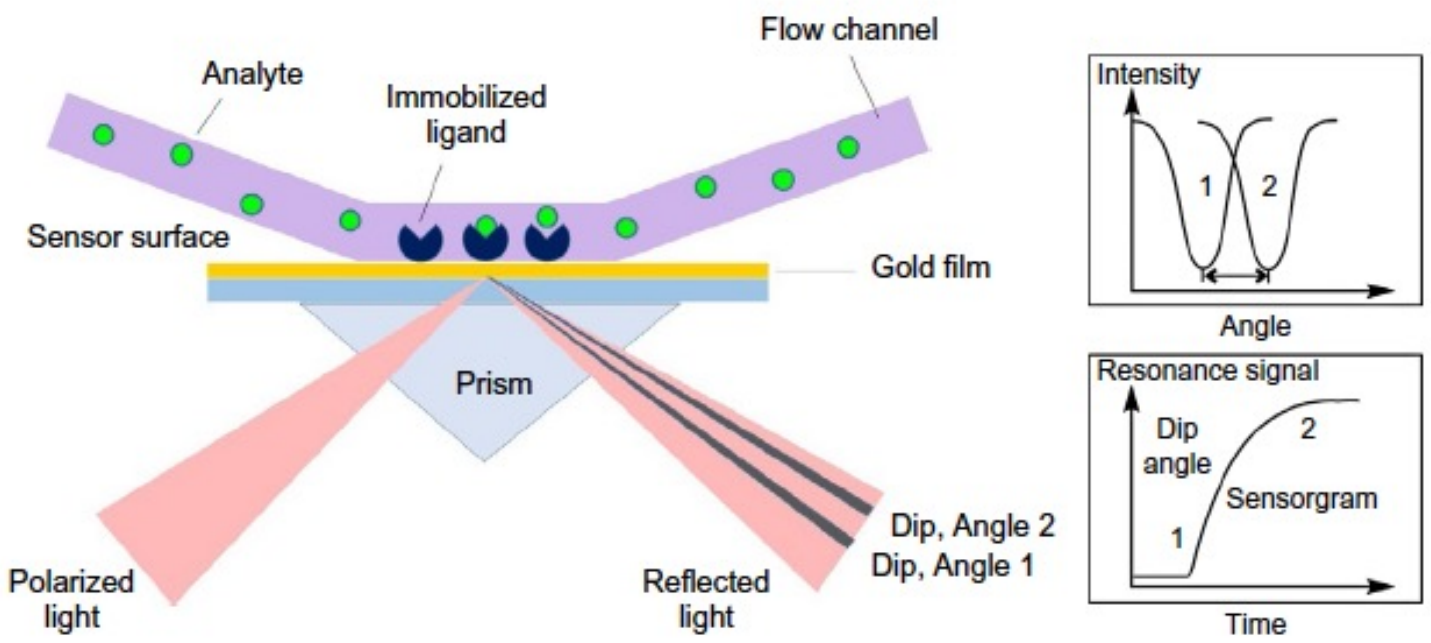


A component of the electromagnetic field of incident radiation (evanescent wave) propagates in the medium with lower refractive index
When the evanescent wave has the correct incident angle, resonance takes place and photons are transformed to plasmons

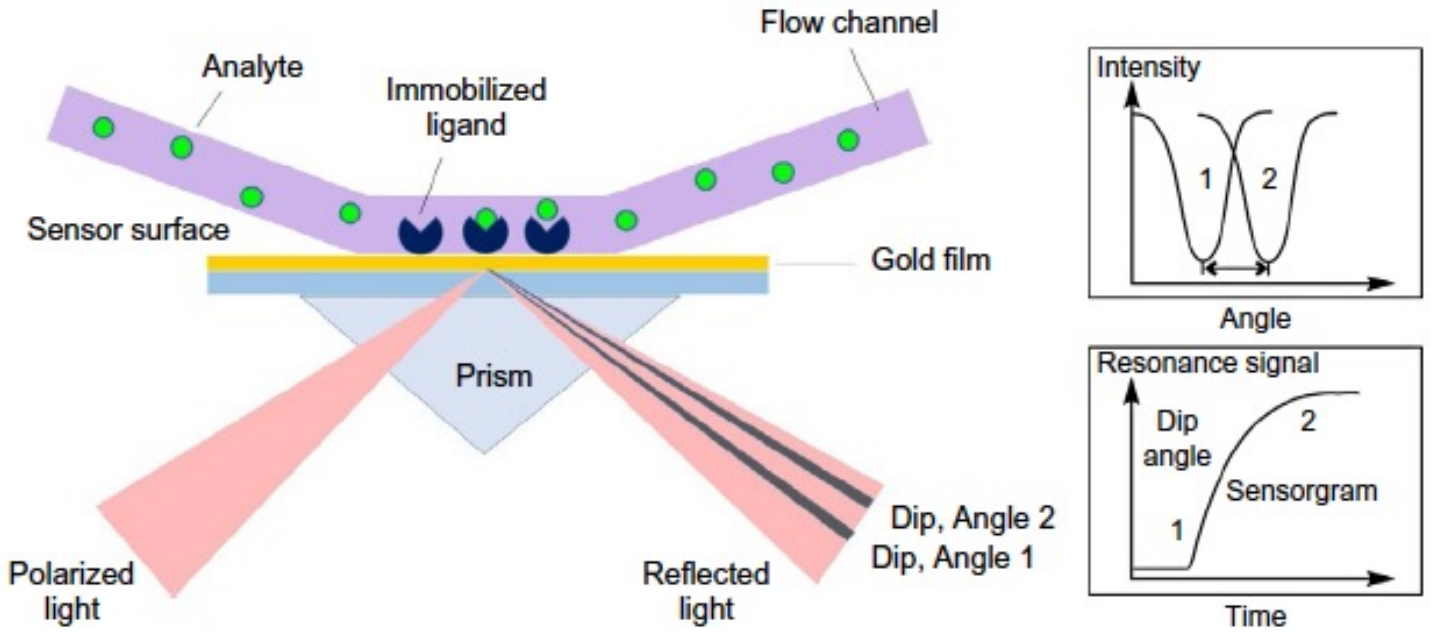
A reduction of intensity of reflected light is observed that produces a dark line in the reflected light beam at a specific angle (SPR angle)

The SPR angle is influenced by:

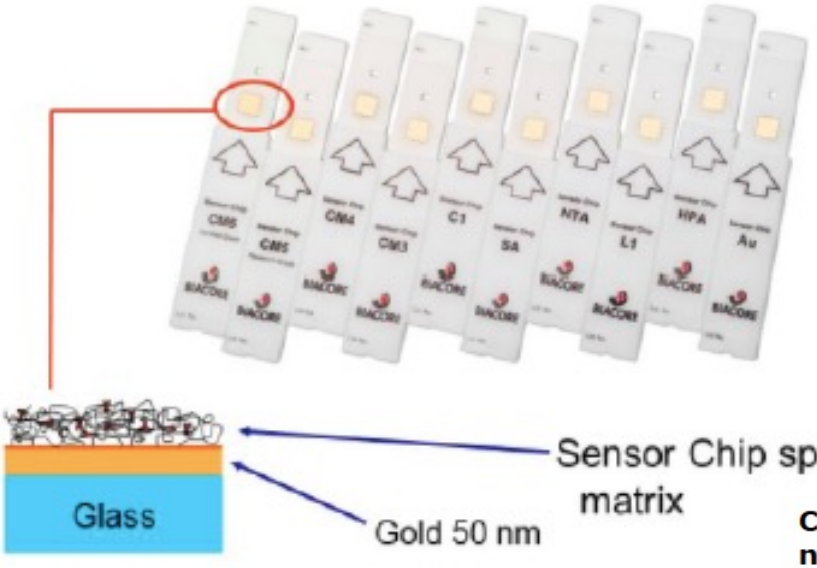
- Features of metallic film
- Wavelength of incident radiation
- **Refractive Index**



The ligand is immobilized on the surface of the sensor chip and the analyte is in solution.
The interaction between ligand and analyte modifies the refractive index of the aqueous layer and causes a shift of the SPR angle that is registered by the instrument.
The refractive index is influenced by the **concentration** of the analyte and by the **dimension** of the analyte at the chip surface

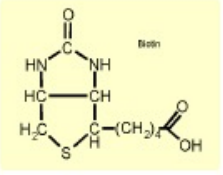


Sensor Chips

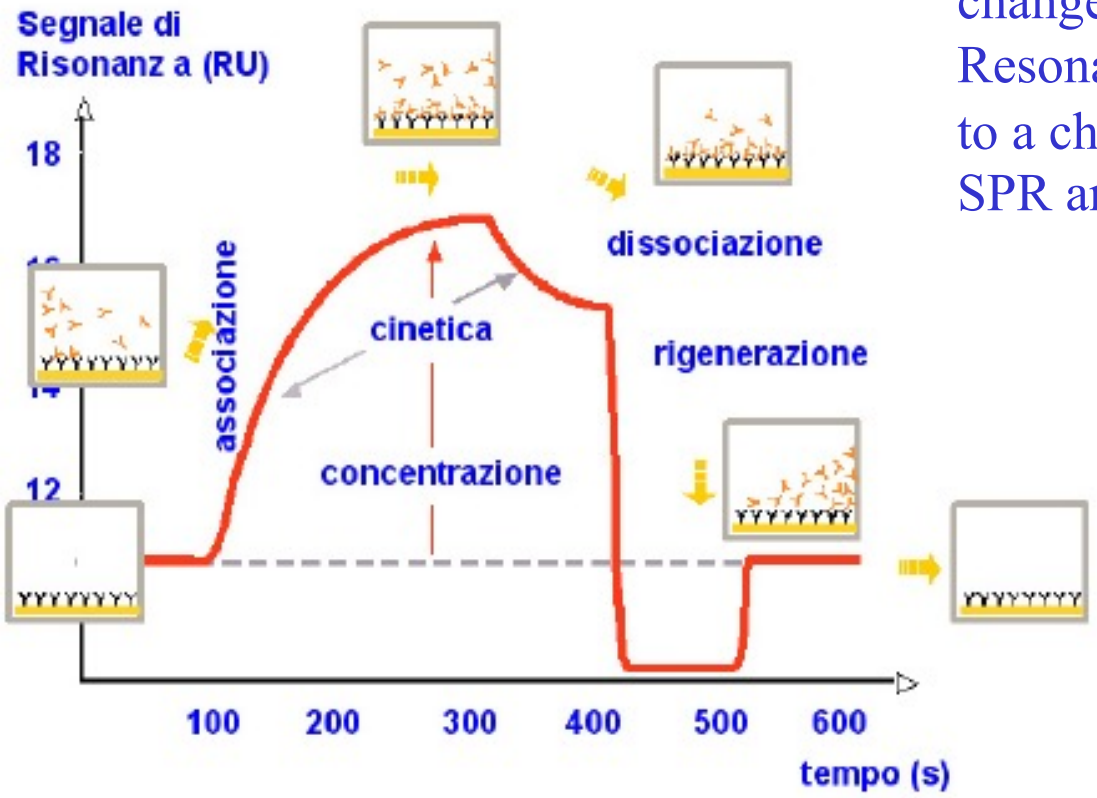


The sensor chip is constituted by a glass support with a thin gold film. The gold film is covered with a matrix for immobilization of the ligand.
The two media with different refractive index are glass and the aqueous solution.

Chip name	Dextran	Modification	Application
CM5	100 nm	100% carboxylation	general
NTA	100 nm	NTA	capturing poly HIS-groups
HPA	none	hydrophobic	lipid capturing
L1	100 nm	lipophilic substances	bilayers to mimic membranes
SA	100 nm	streptavidin	capturing of biotin

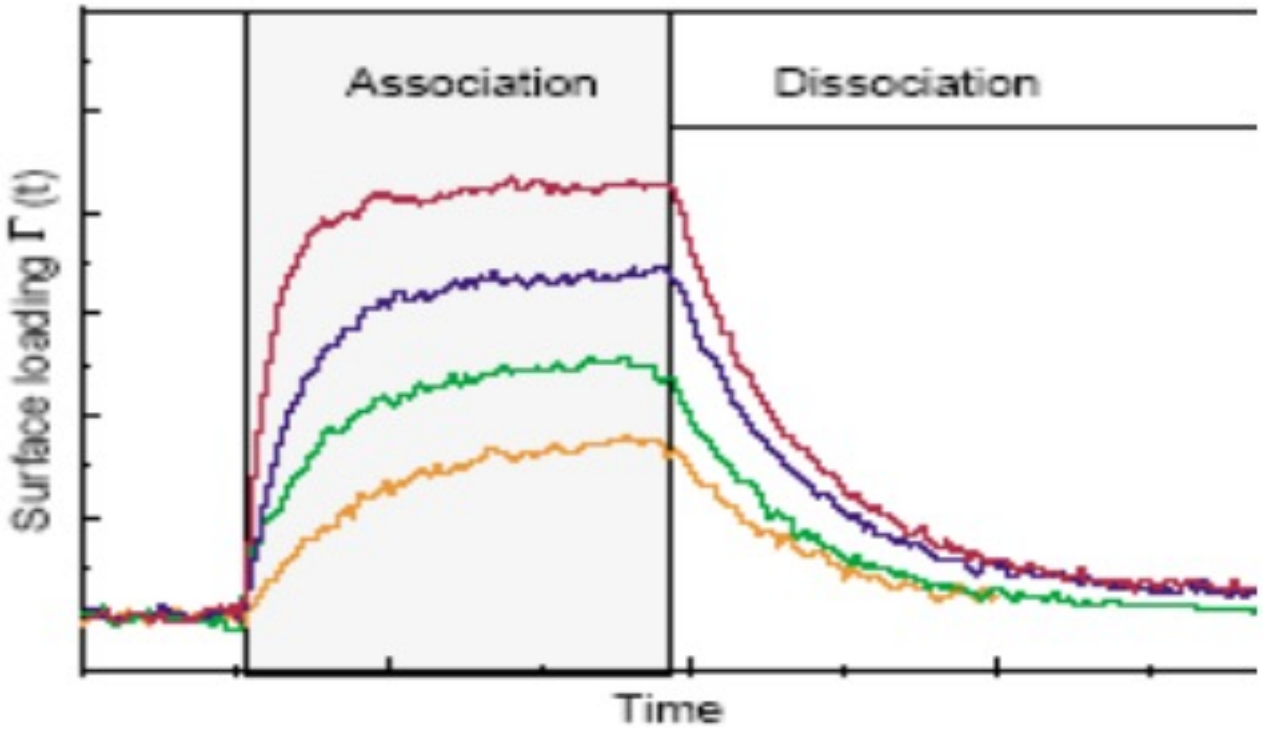


la fase di associazione: l'analita si lega al ligando fino al raggiungimento dell'equilibrio;
la fase di dissociazione: coincide col termine dell'iniezione e fornisce dati utili sulla stabilità del complesso analita-ligando;
la fase di rigenerazione: l'analita viene completamente rimosso dalla superficie del chip.



The optic device measures the change of the SPR angle over time. Resonance units (RU) correspond to a change of 0.0001 ° in the SPR angle.

Generally, different concentrations of analyte are used to obtain k_{on} and k_{off} values from which K_D and K_A can be calculated



Same K_D different k_{on} and k_{off}