



afmb
architecture et fonction
des macromolécules biologiques

Aix*Marseille
université

Approches complémentaires pour l'analyse des
interactions moléculaires.

Alain ROUSSEL

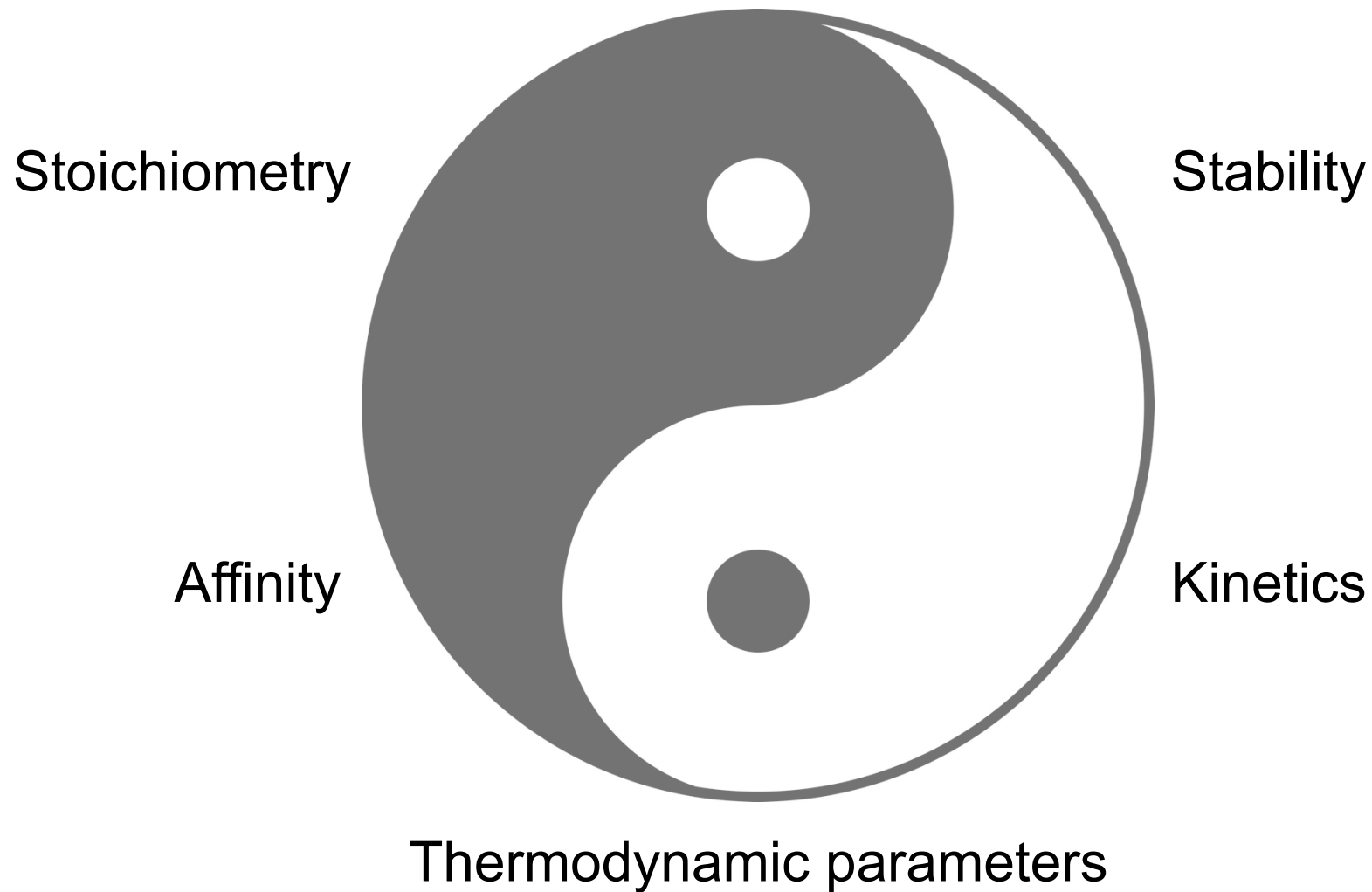


**Ecole Nationale de
Biologie Structurale
Intégrative**

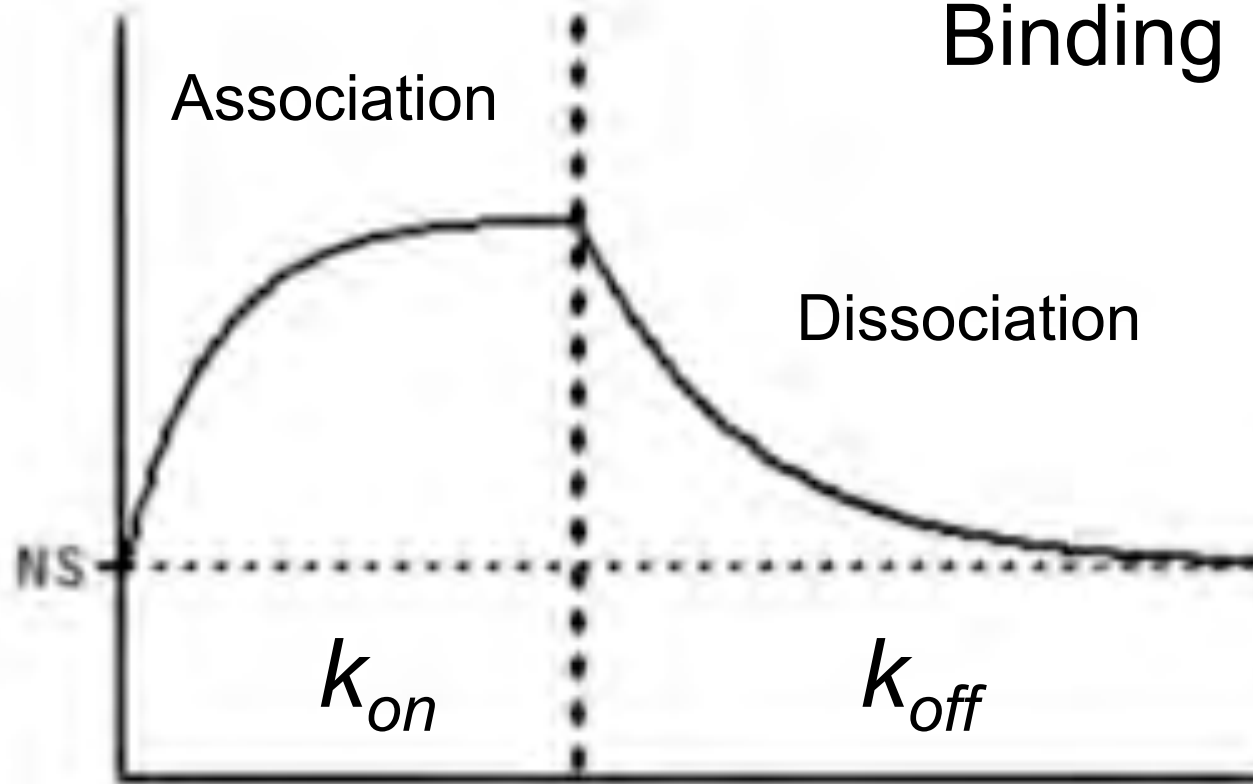
**Juin 2017
Ile d'Oléron**



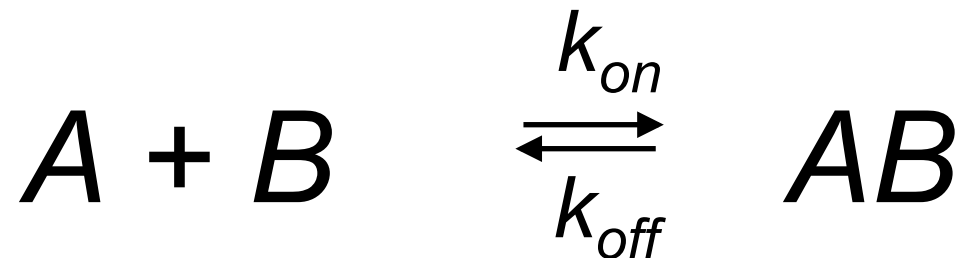
How to characterize a macromolecular complex?



Binding kinetics

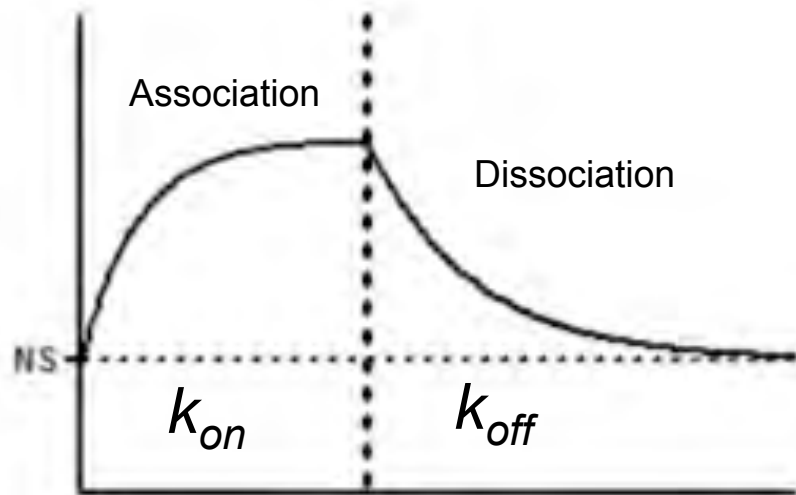


Association: how fast molecules bind = k_{on} ($\text{M}^{-1}\text{s}^{-1}$)

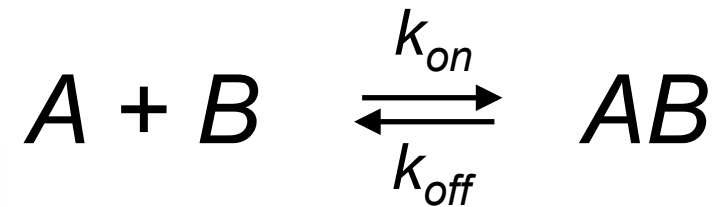


Dissociation: how fast complexes fall apart = k_{off} (s^{-1})

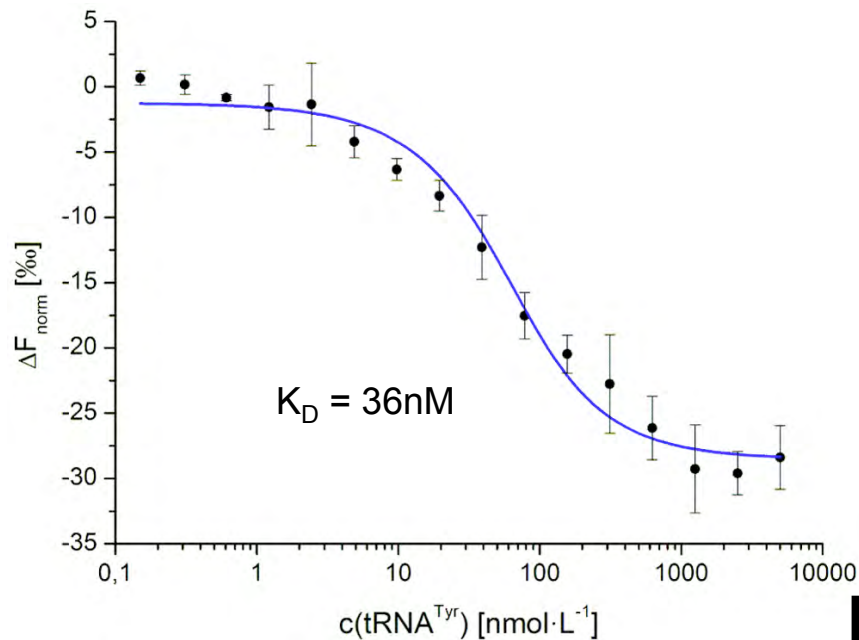
Binding kinetics



Association: how fast molecules bind = $M^{-1}s^{-1}$



Dissociation: how fast complexes fall apart = s^{-1}



Equilibrium

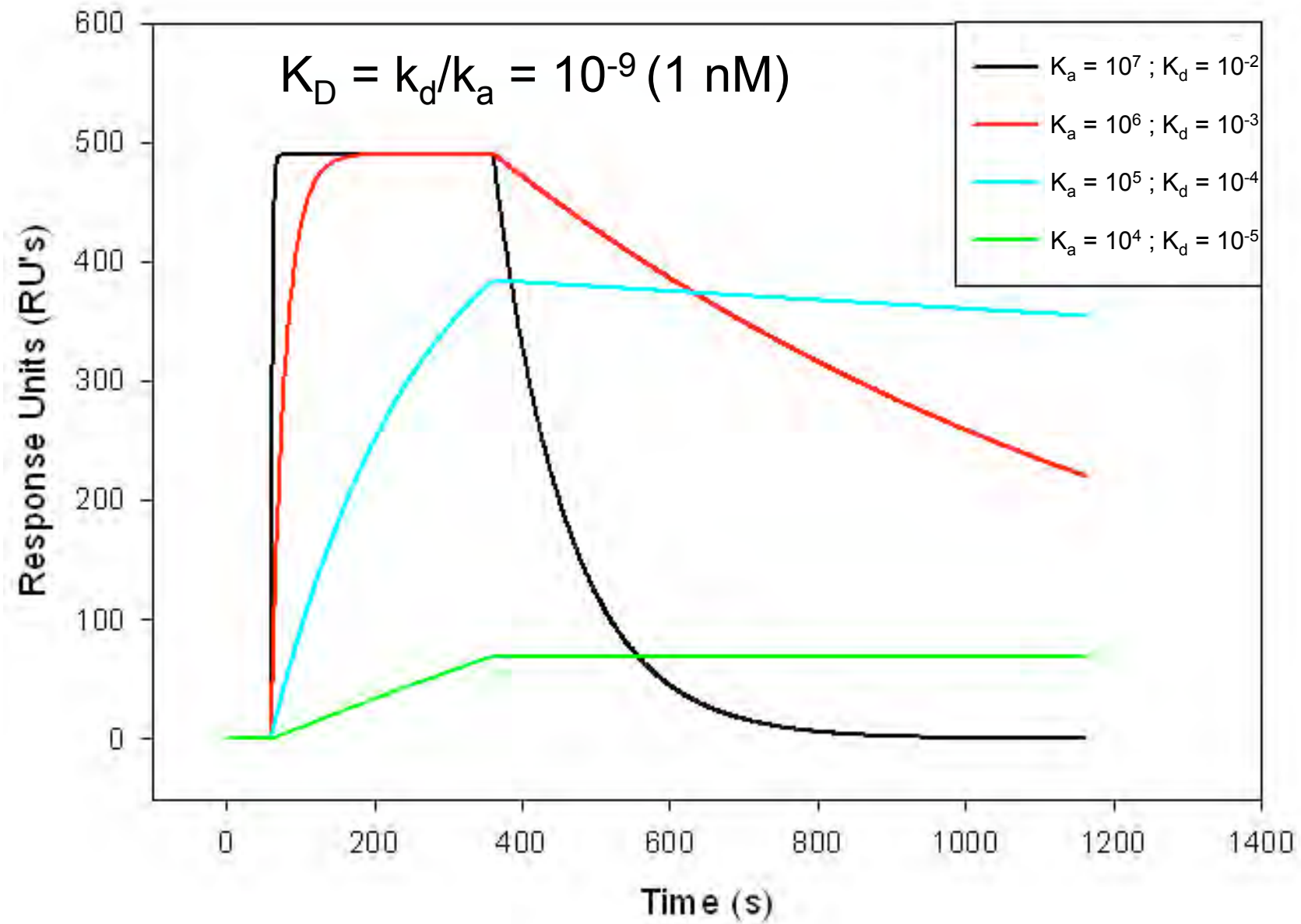
forward binding = backward unbinding

$$k_{on} [A][B] = k_{off} [AB]$$

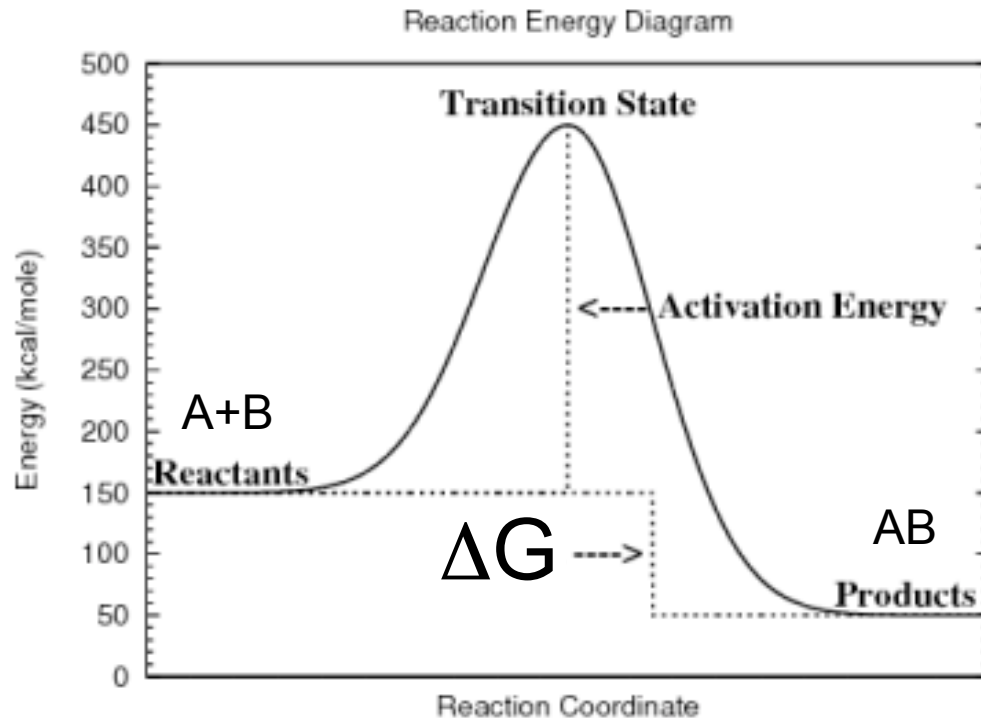
Equilibrium dissociation constant

$$K_D = [A][B] / [AB] = k_{off} / k_{on}$$

Same Affinity ... Different Kinetics



Thermodynamic parameters



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

ΔG : Change in free (Gibbs) energy

ΔH : Change in enthalpy.

It is a measure of the hydrogen bonds and van der Waals contacts involved in the interaction.

Can be measured as heat exchange

ΔS : Change in entropy.

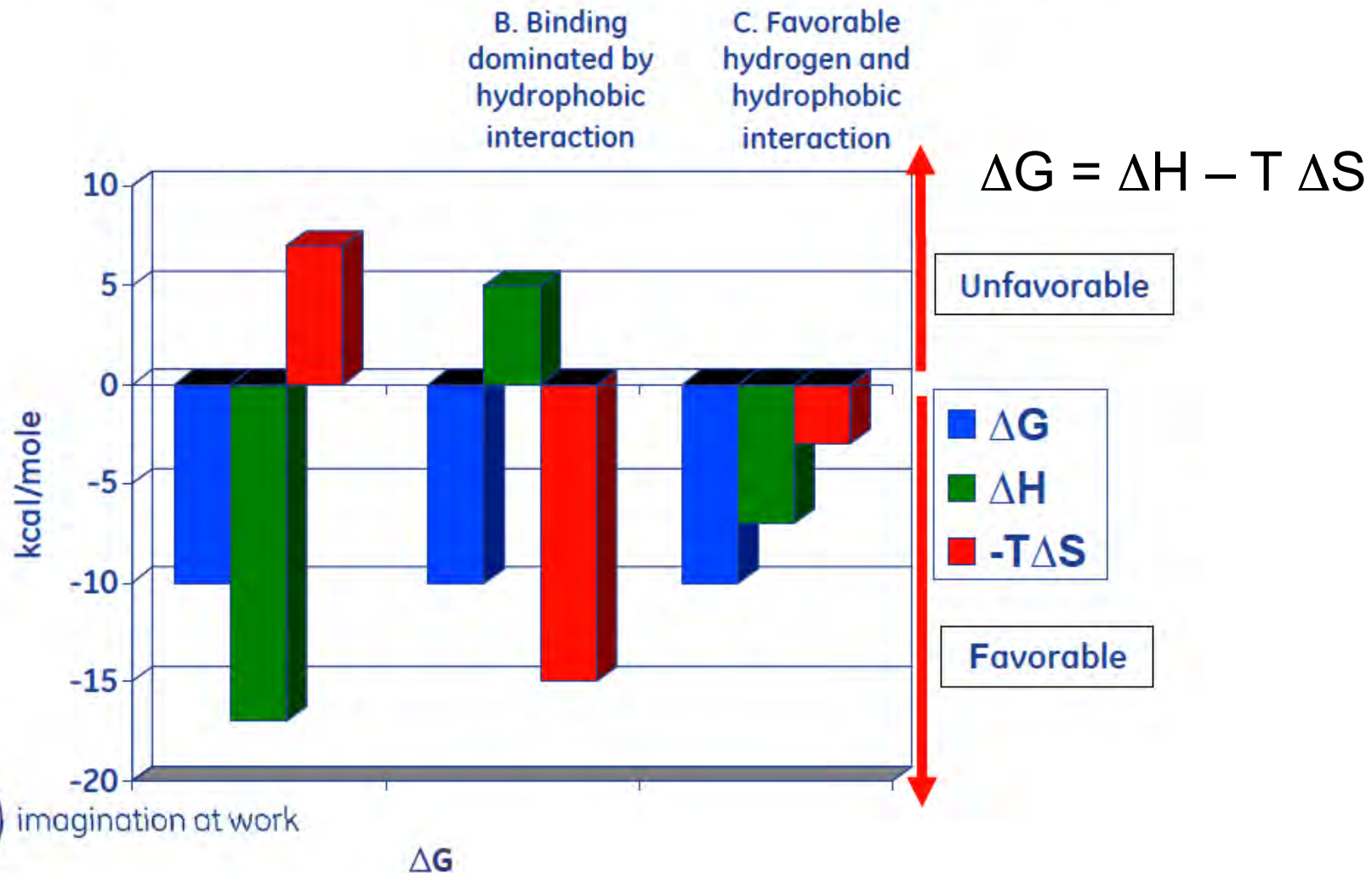
Change in 'chaos': Change in mobility/rigidity conformational changes, solvation (hydrophobic sites)

$$\Delta G = RT \ln(K_D)$$

R is the ideal gas constant, T is the temperature in the Kelvin scale

Affinity is just part of the picture

All three interactions have the same binding energy (ΔG)



imagination at work

Binding equilibria and free energy

$$\Delta G = RT \ln(K_D)$$

Biologically relevant interactions generally have ΔG values in the range of -5 to -10 kcal/mol.

Common language	K_d (M)	K_a (M ⁻¹)	ΔG° (kJ/mol)	ΔG° (kcal/mol)
No affinity (high millimolar)	$> 10^{-1}$	$< 10^1$	> -5.9	> -1.4
Very weak affinity (low millimolar)	10^{-3} to 10^{-1}	10^1 to 10^3	-18 to -5.9	-4.3 to -1.4
Low affinity (high micromolar)	10^{-5} to 10^{-3}	10^3 to 10^5	-30 to -18	-7.1 to -4.3
Moderate affinity (low micromolar)	10^{-6} to 10^{-5}	10^5 to 10^6	-36 to -30	-8.5 to -7.1
High affinity (nanomolar)	10^{-9} to 10^{-6}	10^6 to 10^9	-53 to -36	-13 to -8.5
Very high affinity (pico/femtomolar)	10^{-14} to 10^{-9}	10^9 to 10^{14}	-83 to -53	-20 to -13
Effectively irreversible (low femtomolar)	$< 10^{-14}$	$> 10^{14}$	< -83	< -20

Strength (kcal/mole)*

Biologically relevant interactions use multiple non-covalent interactions to obtain the required affinities.

Bond Type	Length (nm)	In Vacuum	In Water
Covalent	0.15	90	90
Ionic	0.25	80	3
Hydrogen	0.30	4	1
van der Waals attraction (per atom)	0.35	0.1	0.1

Techniques available at the AFMB

Techniques (in order of apparition at the AFMB)	N:M	$\Delta H/\Delta S$	K_D	k_{on}/k_{off}	Stability
FP (Fluorecence)	YES	no	YES	no	yes
TSA (Thermal Shift Assay)	no	no	yes?	no	YES
SPR (Surface Plasmon Resonance)	yes	yes	YES	YES	-
MALS (Multi-Angle Light Scattering)	YES	no	no	no	-
ITC (Isothermal Titration Calorimetry)	YES	YES	YES	yes	-
MST (Microscale Thermophoresis)	YES	yes	YES	no	-
BLI (Bio-Layer Interferometry)	YES	no	YES	YES	-

Other: gel shift, native gel, chromatography, DLS, CD, AUC...

GE Healthcare



Isothermal
Titration
Calorimetry

MicroCal ITC 200

ITC



What happens during an ITC experiment?

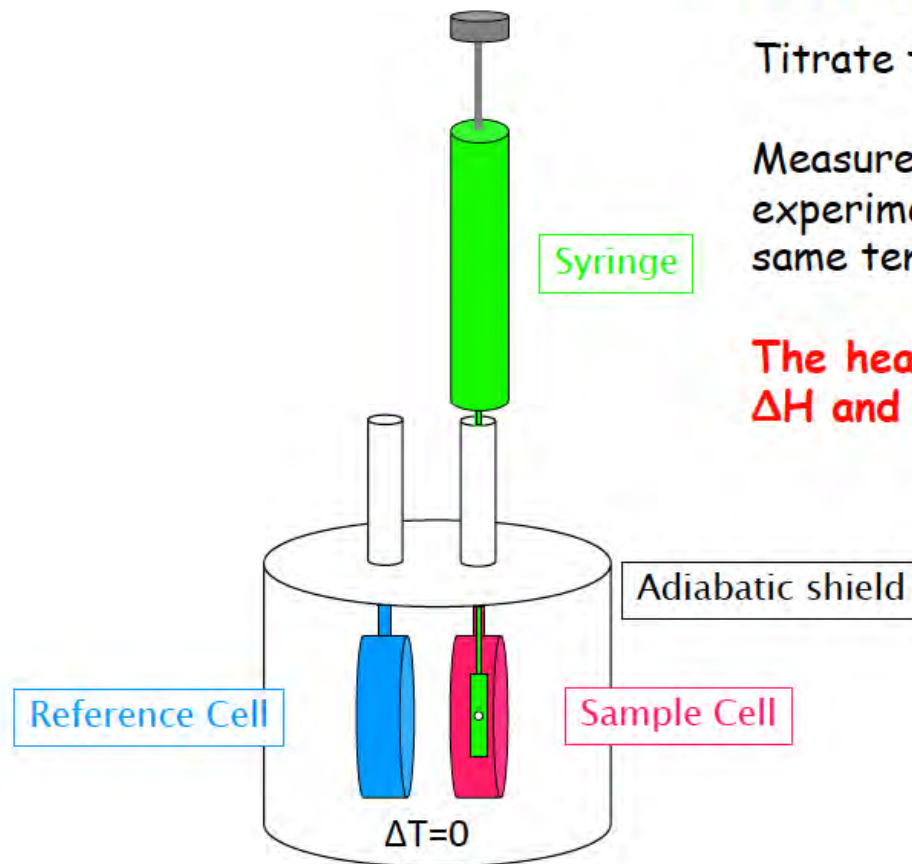
Protein target in sample cell

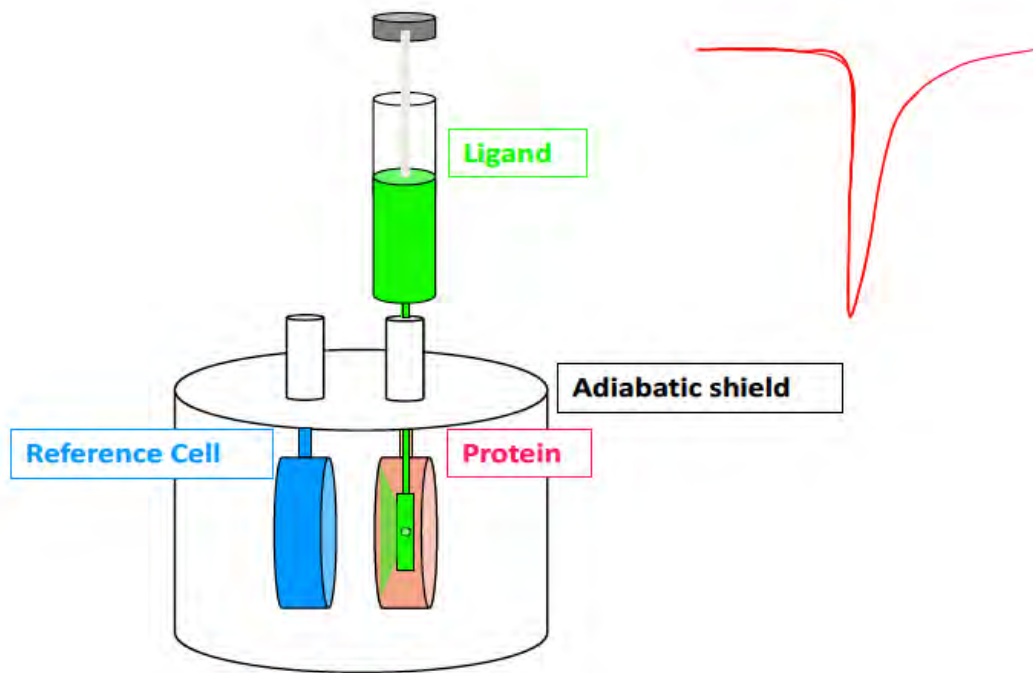
Compound (binding partner) in syringe

Titrate the compound into the protein

Measure the power needed to keep the experiment and reference cells at the same temperature (DP)

The heat provided is proportional to the ΔH and to the amount of complex formed





Endothermique reaction

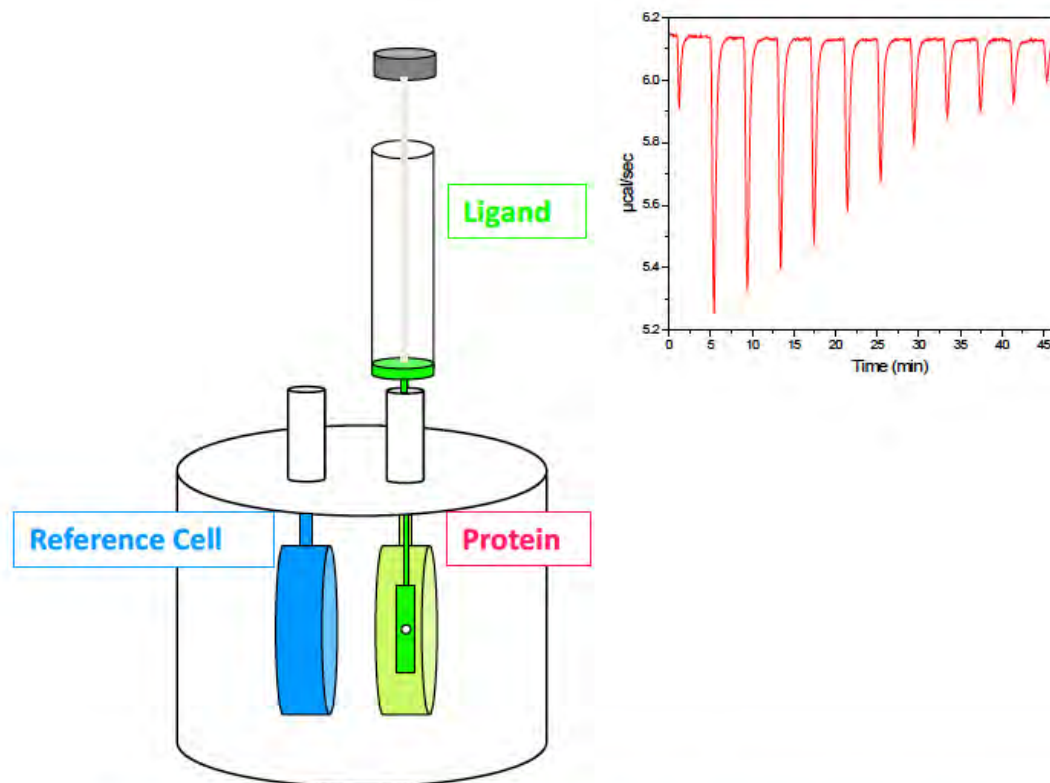
→ Heat **absorbed** in sample cell

→ extra energy will be provided in order to keep $\Delta T=0$

Exothermique reaction

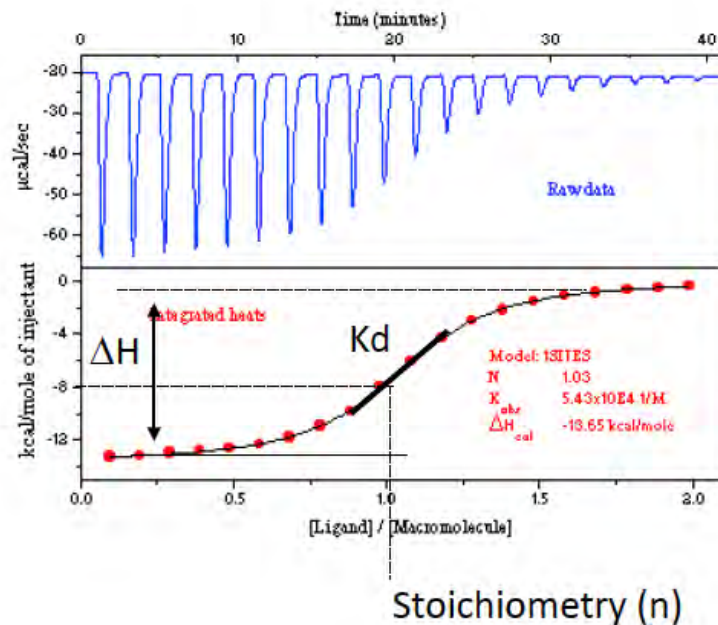
→ Heat **released** in sample cell

→ less energy will be provided in order to keep $\Delta T=0$



- Each peak corresponds to one injection
- During the titration the signal gets smaller because there is less protein available for binding to the ligand
- The area under the peaks is calculated (heat in μcal) and converted to kcal/mol of injectant (ΔH)

Each enthalpy point is plotted against the ratio of [ligand]:[protein]



Data points are fitted according to binding models

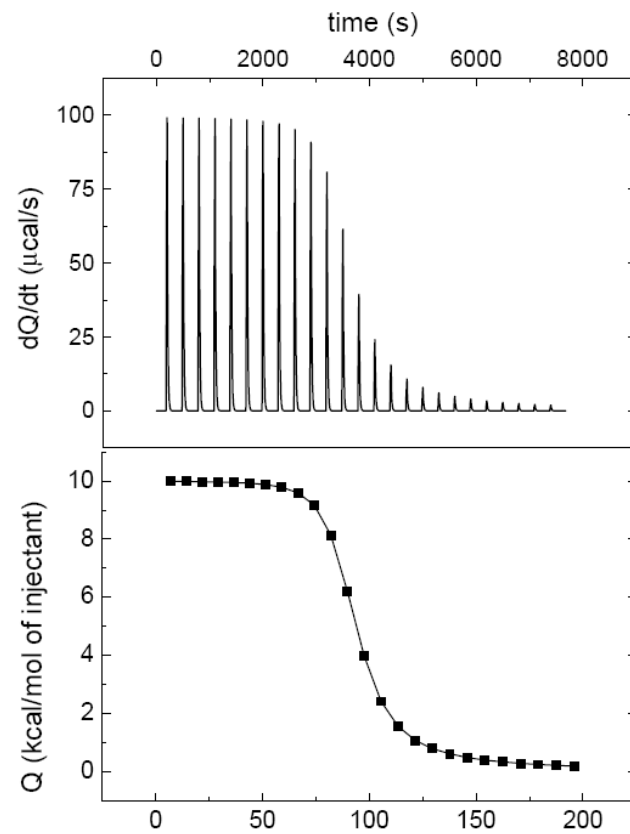
ΔH , n and K_D are calculated from the fitting

ΔG and ΔS are derived from the equations:

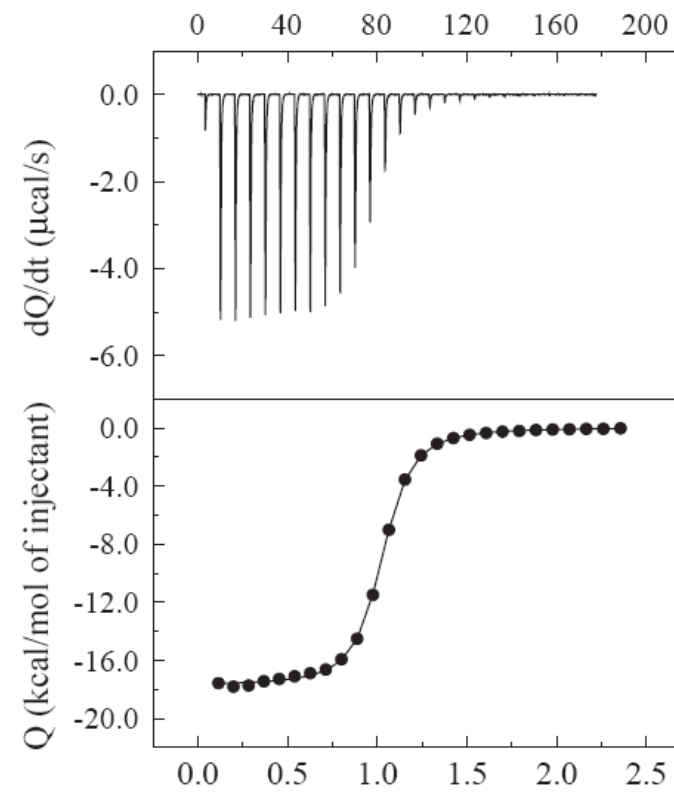
$$\Delta G = RT \ln K_D$$

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

Endothermique



Exothermique





MST

Micro Scale
Thermophoresis



Monolith NT.115

What is Thermophoresis?

Electrophoresis:

We apply an electric field

We separate the molecules by charge (and also size)

Thermophoresis:

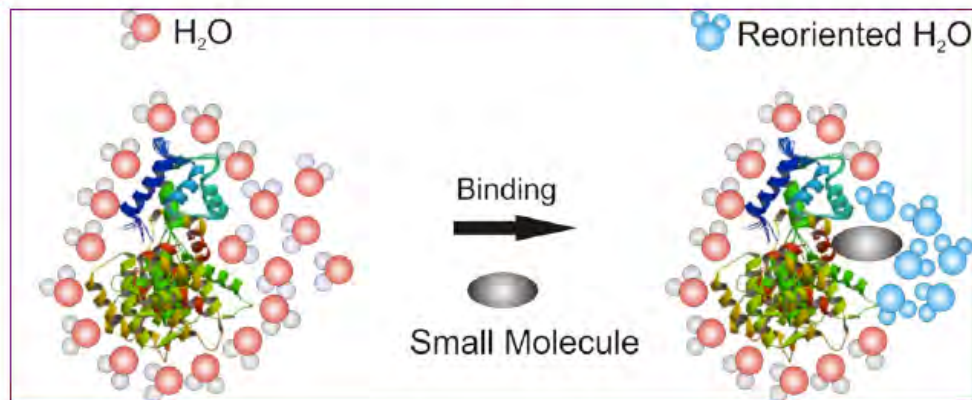
Sample is in solution in a capillary

An infrared laser generates a temperature gradient (between 1-6 K)

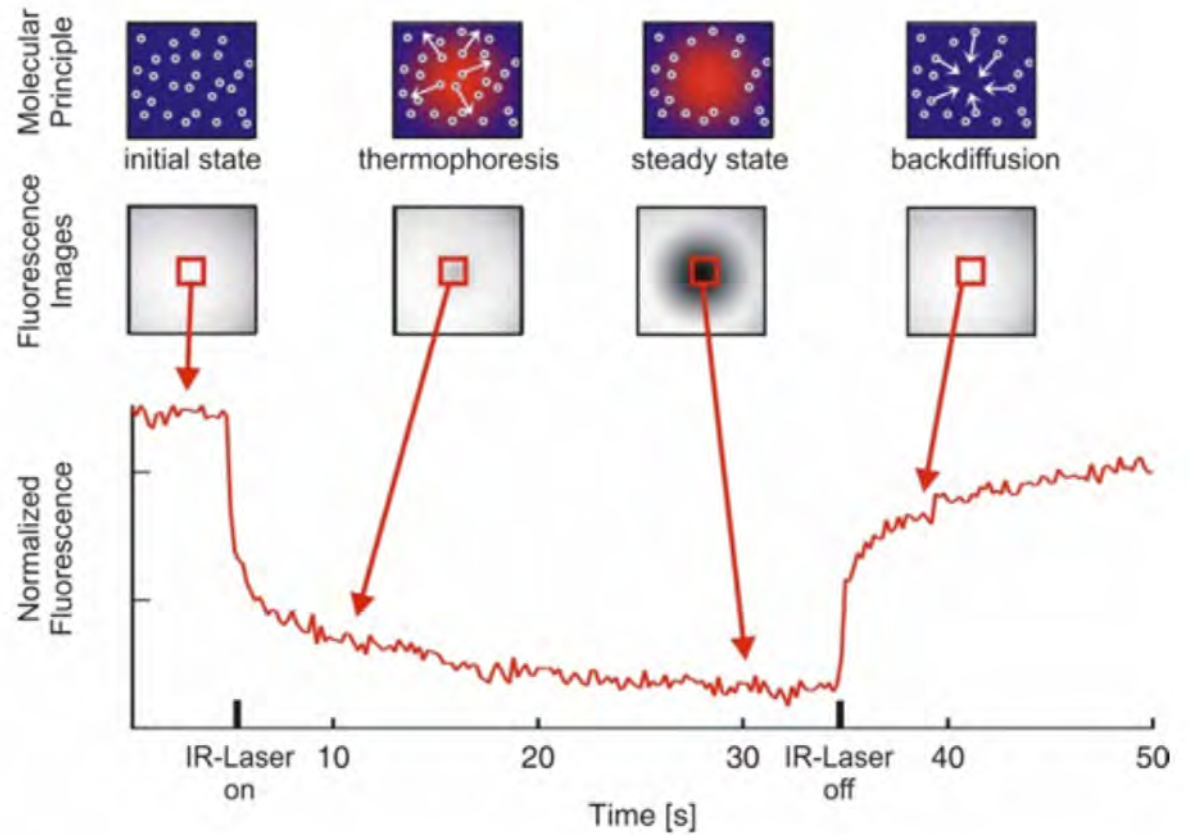
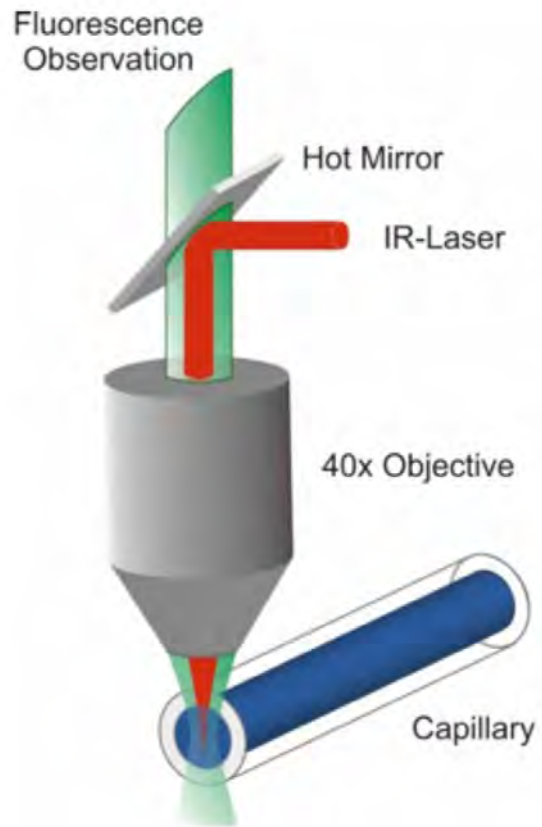
Thermophoresis is the motion of molecules in temperature gradients.

Movement is detected by through **fluorescence of one of the binding partners.**

Thermophoresis depends on size, charge, solvation entropy and conformation of the molecule.



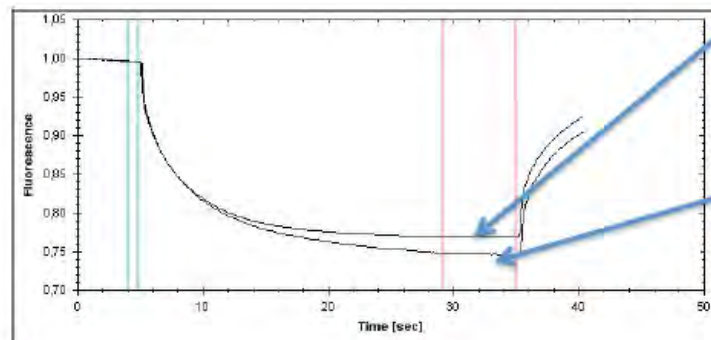
Thermophoresis experiment





- 16 capillaries
- Concentration of the fluorescent partner kept constant
- Titration of the non-fluorescent partner

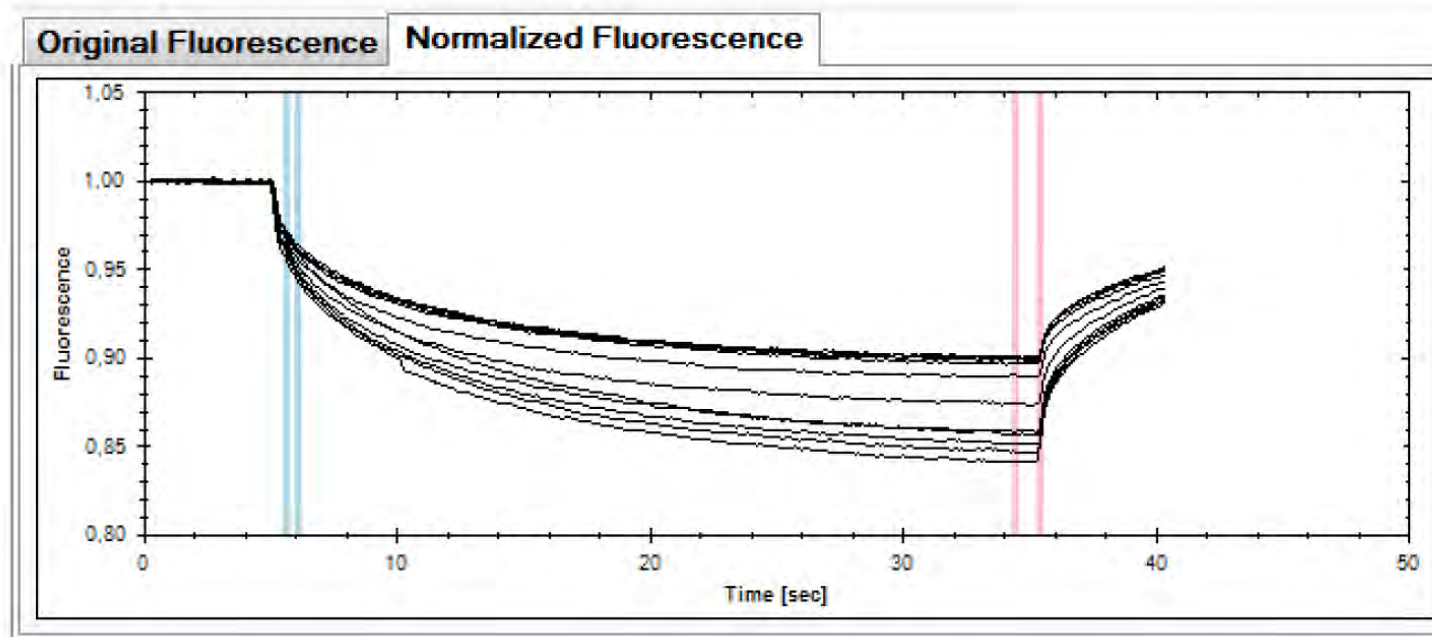
Thermophoresis measurement (MST) on first capillary (highest concentration of ligand)



MST on second capillary

Depending on the level of complexation, the labelled molecules will move differently on the temperature gradient.

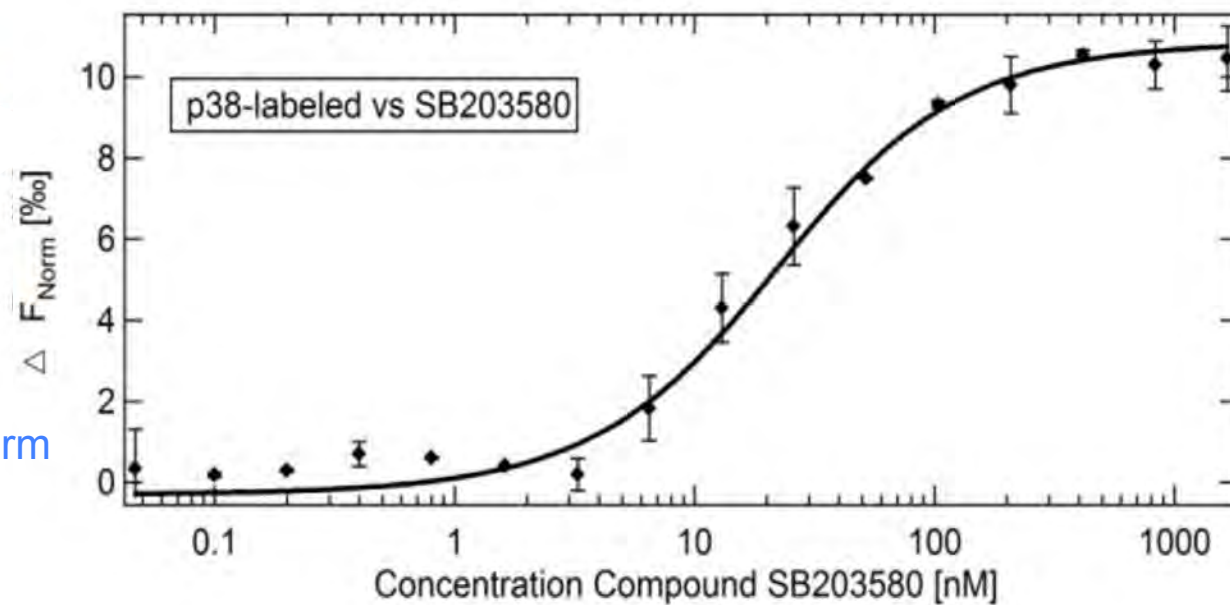
Thermophoresis will depend on size + charge + hydration shell + conformation



Measurement
over 16
capillaries

$$F_{\text{norm}} = F_{\text{conc}} - F_0$$

$$\Delta F_{\text{norm}} = F_{\text{norm}} - F_{\text{norm}}$$



The fluorescent partner must be monodisperse and should not stick to the capillaries

In most cases you will need to add additives to the buffer: detergent (0.05% Tween 20) BSA, L-arginine, glycerol...

Ionic force and pH must be adapted to the interaction

Chose the right type of capillary: standard, coated (hydrophilic, hydrophobic)

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SPR

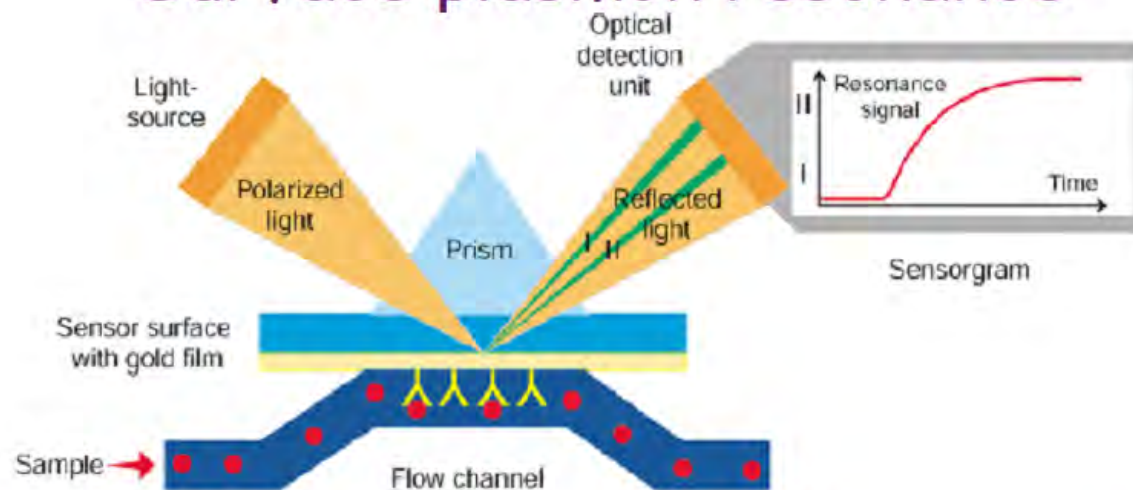


Surface
Plasmon
Resonance

Biacore T200



Surface plasmon resonance



Physical phenomenon: Surface plasmon resonance

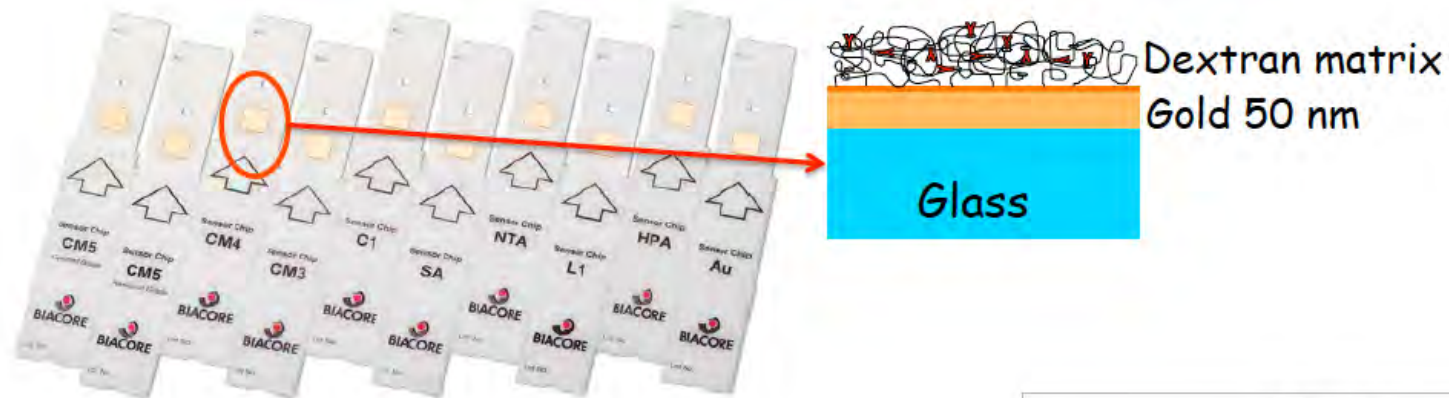
SPR machine detects refractive index changes close to the sensor surface

On the sensor surface we will immobilize one of the components (LIGAND)
The other component (ANALYTE) will be injected in a continuous flow.

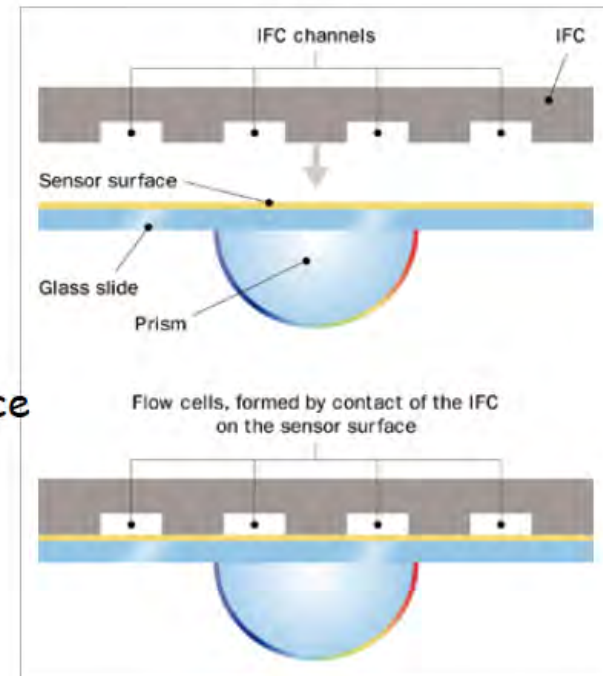
Binding of the analyte will change the refractive index near the surface, this change will generate a resonance signal that will be measured in real time and represented in a sensorgram

A sensorgram represents the resonance signal (in resonance units RU) as a function of time. This response is proportional to the mass retained near the surface

Sensor Chips and flow cells

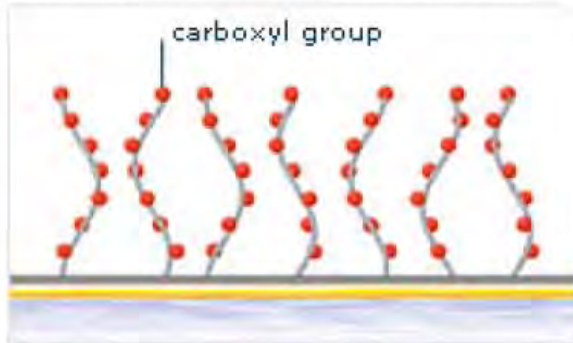


Flow cells are formed by pressing an integrated microfluidic cartridge (IFC) against a sensor surface

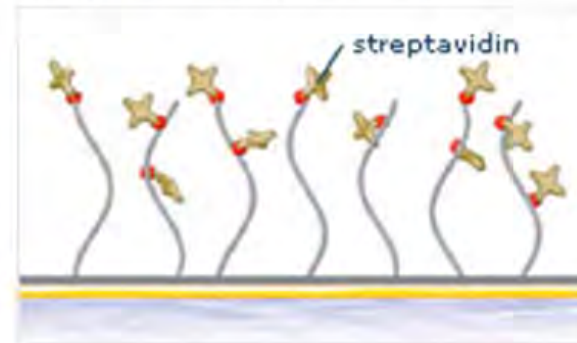


Different types of sensor chips

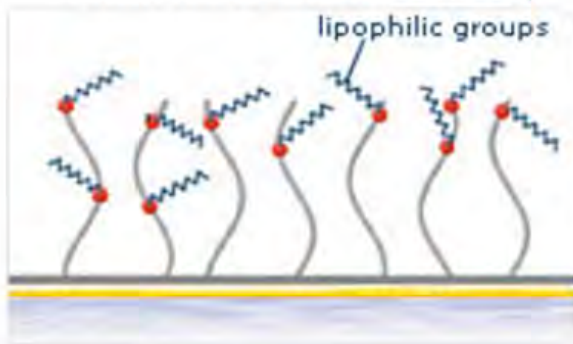
CM5 (carboxymethyl-dextran) Coupling
via -NH₂, -SH, -CHO, -OH or -COOH



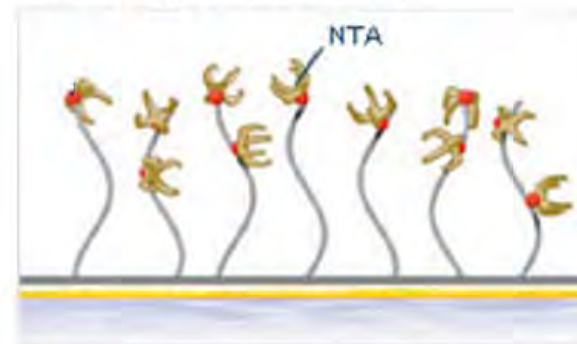
SA (immobilization of
biotylated molecules)



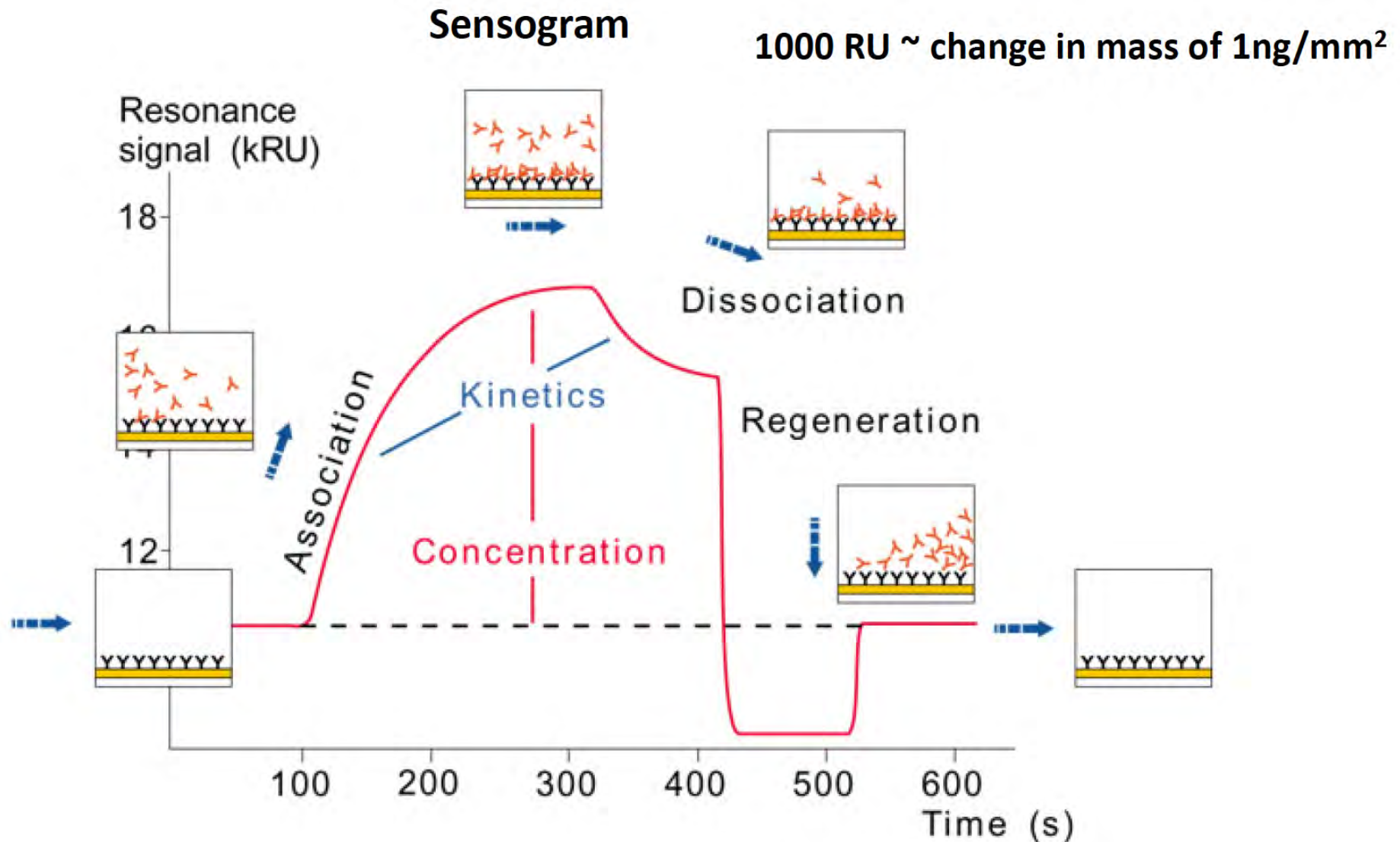
L1 (immobilization
of membrane structures)



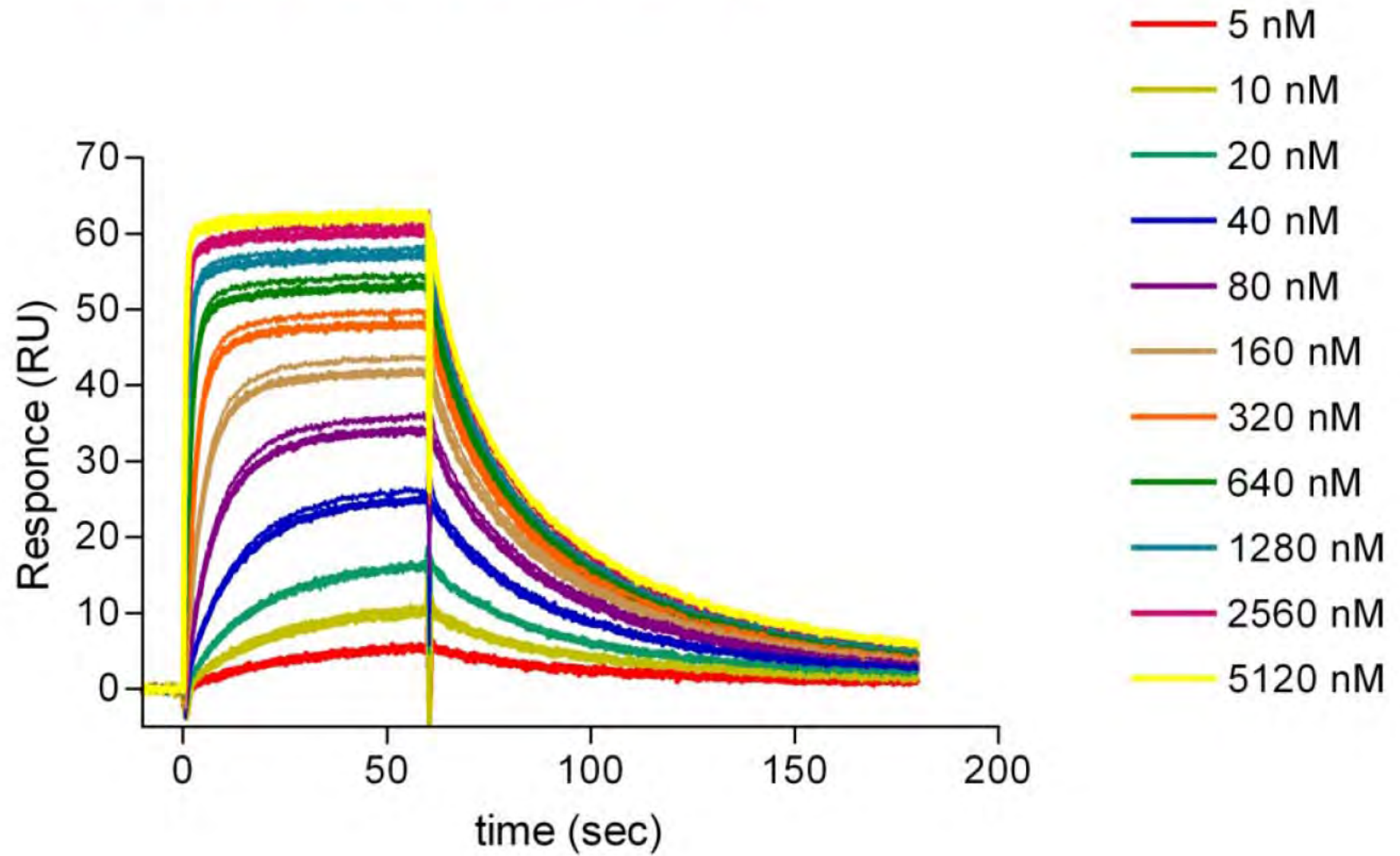
NTA (binding of Ni²⁺ /
his-tagged proteins)



Typical binding kinetic's experiment



Example of binding



How to set up an SPR experiment?

Ligand Immobilization

- Choice of Immobilization chemistry (covalent, via a His-tag, biotin/streptavidine)
- Stability of ligand
- Level of immobilisation (enough but not too much...)

Kinetic analysis: immobilize low amount of ligand to avoid rebinding of analyte during dissociation

Analyte Binding

- Choice of running buffer (pH, ionic strength, detergent...)
- MW of the analyte (small molecules may be difficult to analyse)
- Availability of the analyte

Regeneration

- Regeneration solution (ligand/analyte-dependent): detergent, pH, salt...
- Stability of the ligand

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A Division of **Pall Life Sciences**

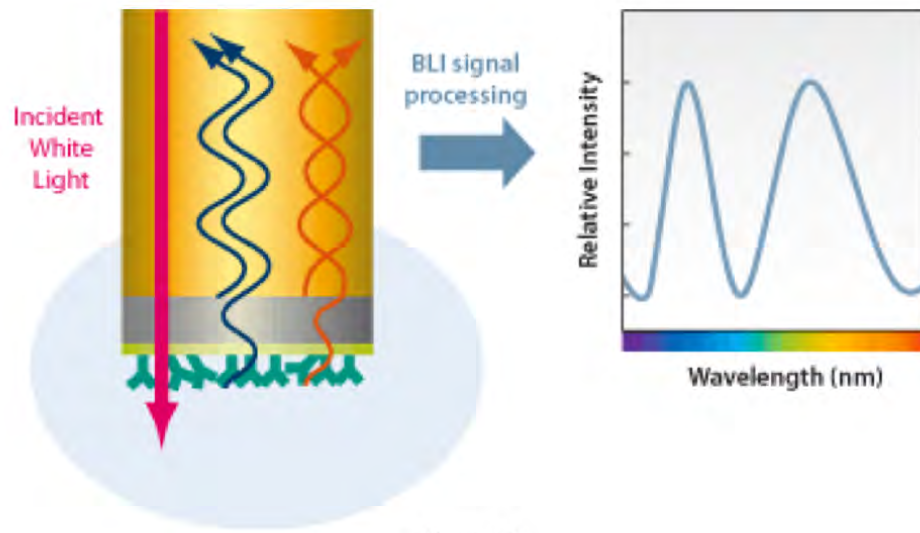
BLI

Bio-Layer
Interferometry

Octet Red 96



Bio Layer Interferometry



Bio-Layer Interferometry (BLI) is an optical analytical technique that analyzes the interference pattern of white light reflected from two surfaces: a layer of immobilized protein on the biosensor tip, and an internal reference layer.

Bio Layer Interferometry

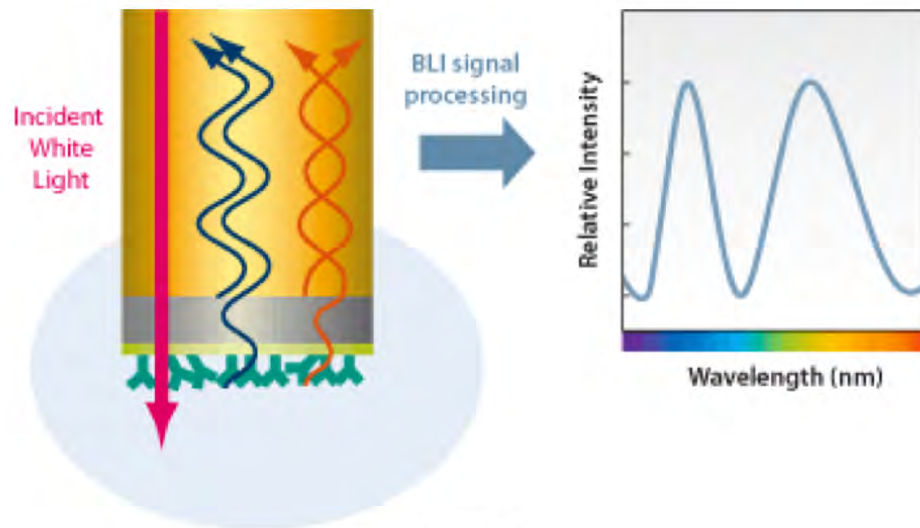
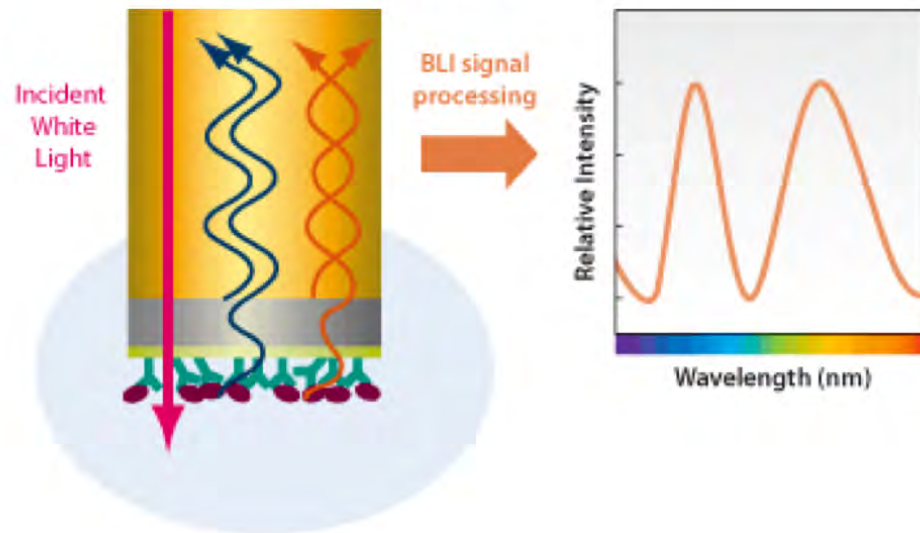


Figure 1



Bio-Layer Interferometry (BLI) is an optical analytical technique that analyzes the interference pattern of white light reflected from two surfaces: a layer of immobilized protein on the biosensor tip, and an internal reference layer.

Any change in the number of molecules bound to the biosensor tip causes a shift in the interference pattern that can be measured in real-time

Bio Layer Interferometry

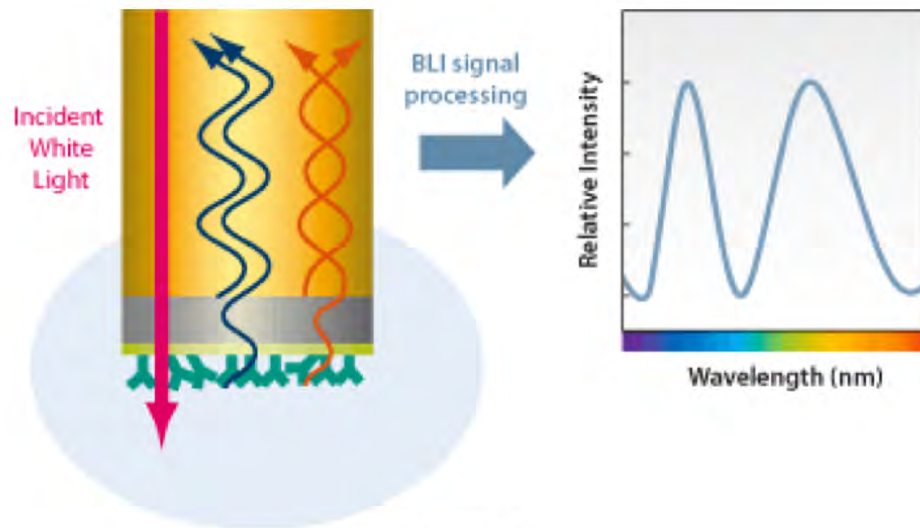
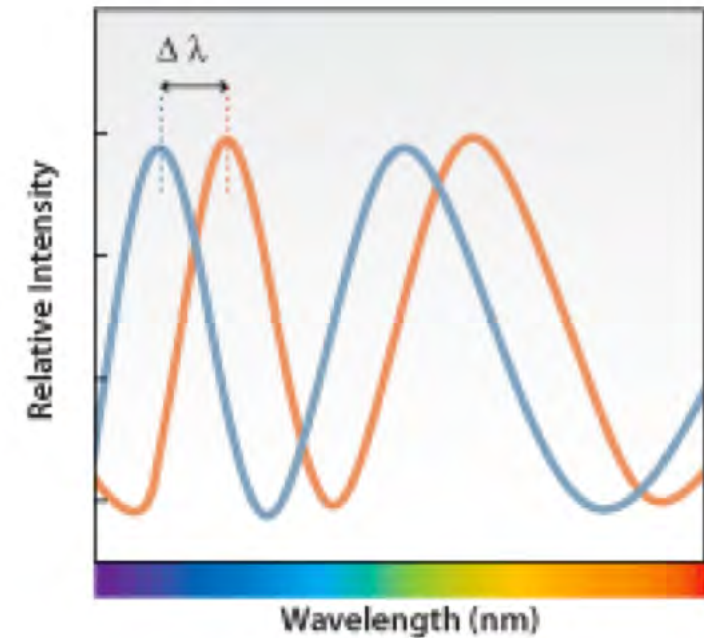
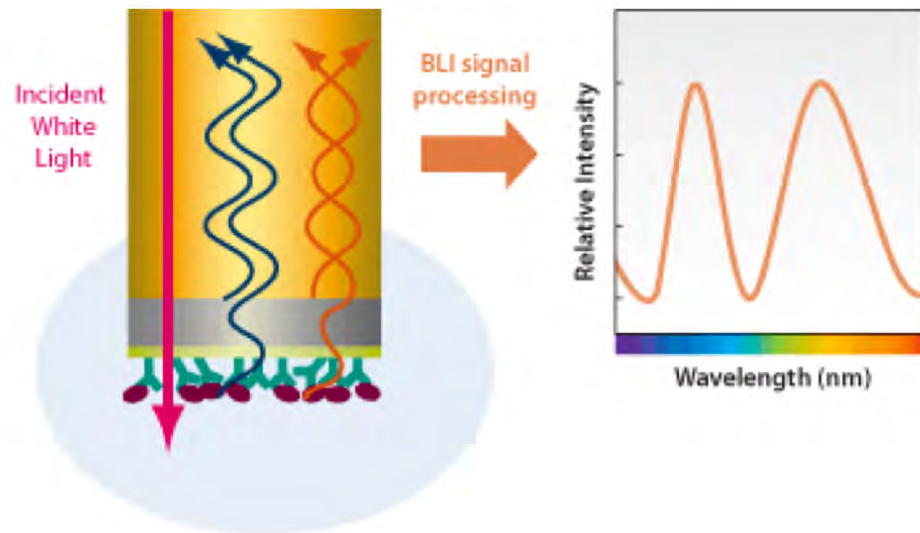
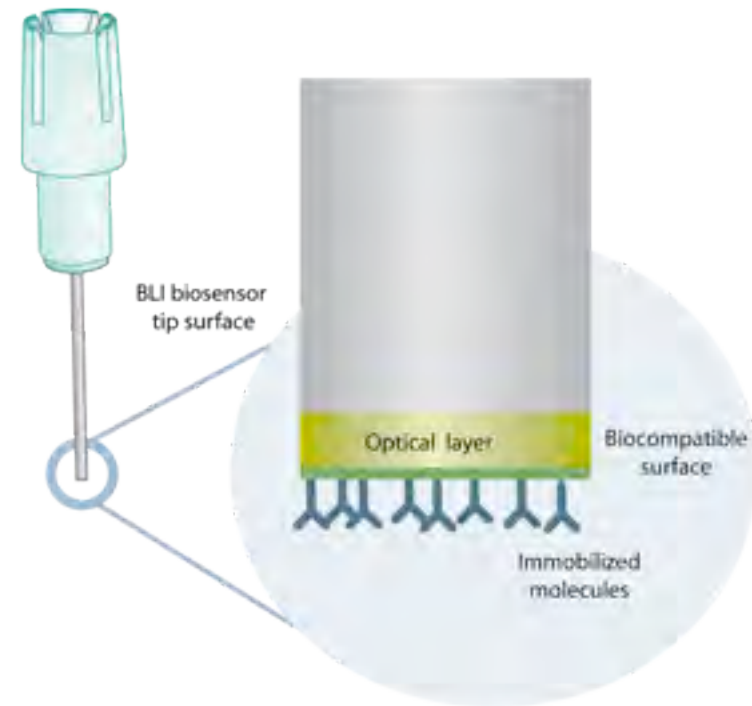
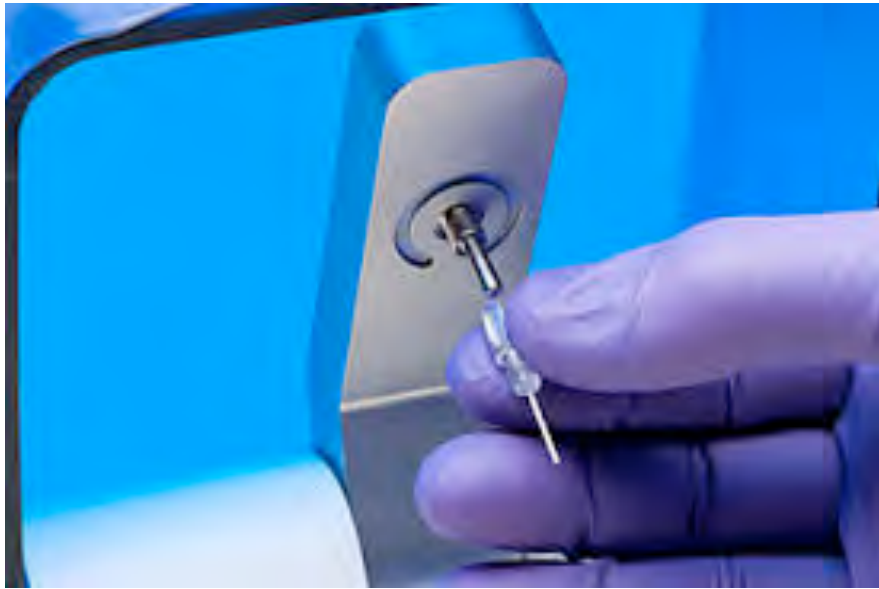


Figure 1



The binding between a ligand immobilized on the biosensor tip surface and an analyte in solution produces an increase in optical thickness at the biosensor tip, which results in a wavelength shift, $\Delta\lambda$, which is a direct measure of the change in thickness of the biological layer.

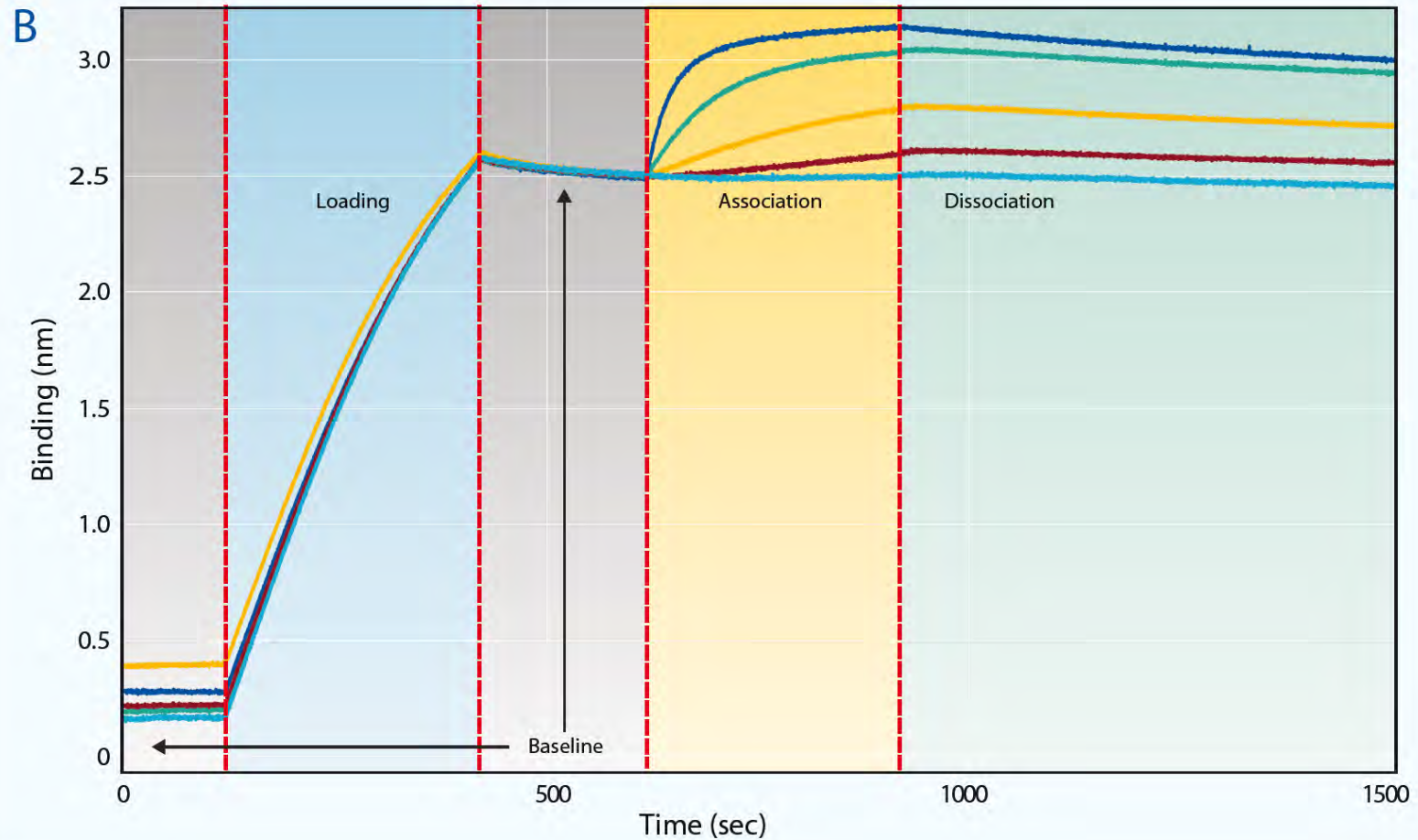
Dip and Read™ Biosensors



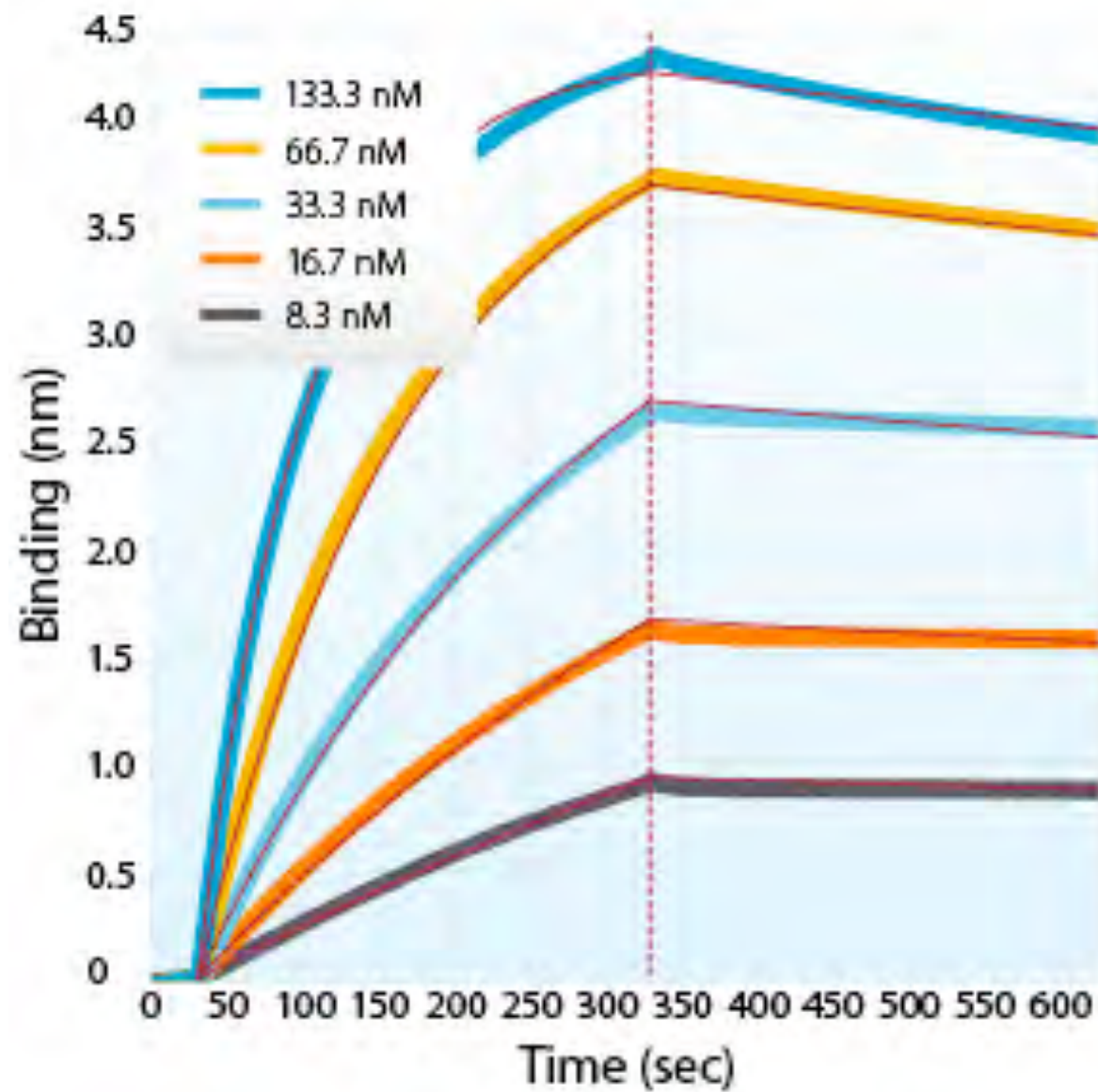
Direct immobilisation: amine coupling, biotin intercation with streptavidin

Capture-based approach: high affinity capture antibody (anti-FC, anti-His) or use of known motif or tag (protein A, Ni NTA)

Typical binding kinetic's experiment



Example of binding



ITC	MST	SPR	BLI
Affinity Thermodynamics	Affinity	Affinity Kinetics	Affinity Kinetics
Interaction in solution No labeling is required No limit on molecular weight	Easy to use No limit on molecular weight	Low sample amount Measure in any buffer No limit on molecular weight	Fast and Easy to use Measure in any buffer (culture media) No limit on molecular weight
Big amount of protein High sample concentration Buffers must match exactly	Interaction capillaries The labelled protein must be really monodisperse	Microfluidics Ligand immobilisation Regeneration Non specific interaction with sensor chip	Ligand immobilisation (Regeneration) Non specific interaction with bio sensor surface
Cost 80k€ No maintenance	Cost 80k€ No maintenance	Cost 300k€ Expensive maintenance	Cost 150k€ No maintenance
Fragile syringe	Capillaries	Sensor chips	Bio-sensors

Which one is the best?

It depends on:

- The question you want to answer
- The amount of material you have
- Time...

They all have advantages et disadvantages

Very important

be aware of the limitations

never try to over-interpret your results

How much protein do I need?

For all techniques

Purity is crucial for obtaining quantitative reliable results.

« Real » concentration must be measure as accurately as possible

ITC Protein 300µl concentration = $10 \times K_D$
Ligand 60 µl concentration = $100 \times K_D$

Biacore Ligand, depends on immobilisation 50-400 nM, 100µl
Analyte, titration between 0.1 and $10 \times K_D$
Quantity depends on contact time

Thermophorèse Labeled protein 100 µl 20 µM
Ligand 20 µl concentration = $40-50 \times K_D$

BLI Ligand, depends on immobilisation 50-400 nM, 200µl
Analyte, titration between 0.1 and $10 \times K_D$, 200µl

Survey of the year 2007 commercial optical biosensor literature

Rebecca L. Rich^a and David G. Myszka^{a*}

Abstract:

In 2007, 1179 papers were published that involved the application of optical biosensors. We found a disappointingly low percentage of well-executed experiments and thoughtful data interpretation. We are alarmed by the high frequency of suboptimal data and over-interpreted results in the literature....

In fact, a problem in most of the published data we see is that the authors apparently did only one experiment; it looks like they walked up to the machine, chucked in their samples, and **published whatever data came out**.

Many users who generate poor-quality data are either **too ignorant** to recognize the problem **or too lazy** to want to fix it.

Study on the interactions between antibodies and antigens : a comparison between SPR and BLI methodologies

Christine Kellenberger



Emmanuel Nony



Hans de Haard





Biacore T200

CONSUMABLE
sensor chips GE (pack of 3) about 450 €



4 channels

MAINTENANCE
use of 0,1um filtered buffers
weekly and monthly
(desorb, sanitize etc)
MICROFLUIDIC replacement every 6 months
Total annual maintenance > 20 K€



Octet Red 96

CONSUMABLE
biosensors
tray of 96 : 500-700 €
large choice of fonctionnalités

NO MAINTENANCE



Experimental protocols for determination of kinetic constants



Biacore T200 (platform)

« Standard » protocol

- Ab immobilization
- choice of sensor chip : CM5 type => amine coupling
- pH scouting for Ab immobilization
- immobilization of Ab using target level wizard
- for theoretical R_{max} 50-100 RU
- regeneration conditions (to dissociate Ab-Ag complex)
- repetition of injections of Ag (+regeneration)

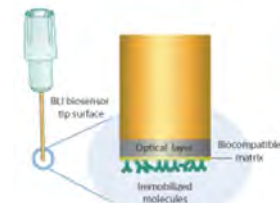


Sensor Chip CM5, series S (1 of 1)

OctetRed96

development of protocol

- Ab immobilization
- choice of biosensor :
streptavidin, Ab biotinylation (ratio 1:1)
AHC (anti human IgG Fc)
- loading of Ab on 8 biosensors
- loading conditions (5-10 $\mu\text{g/ml}$)
about 1 to 1.5 nm
- Ag as analyte in 200 μl wells
(can be recovered)

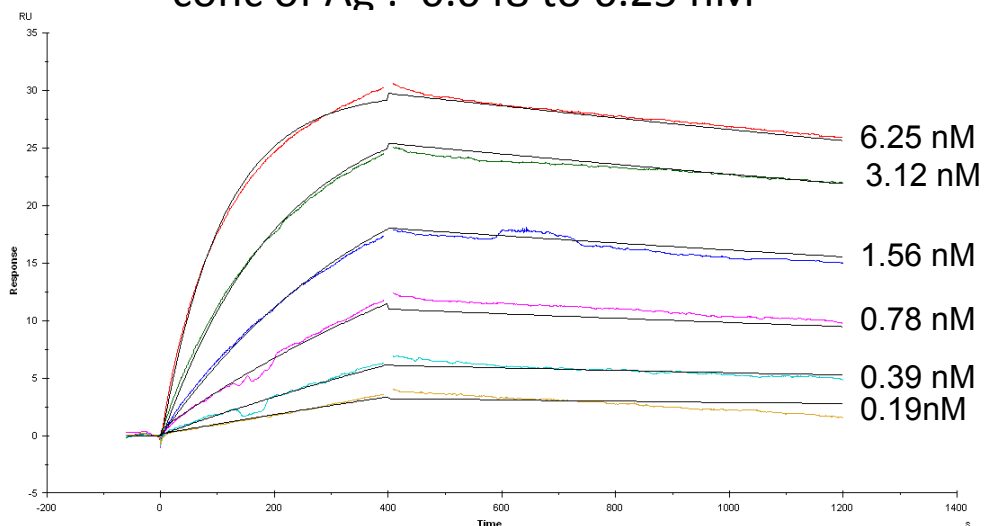




Kinetics constants for Example 1 (same K_D)



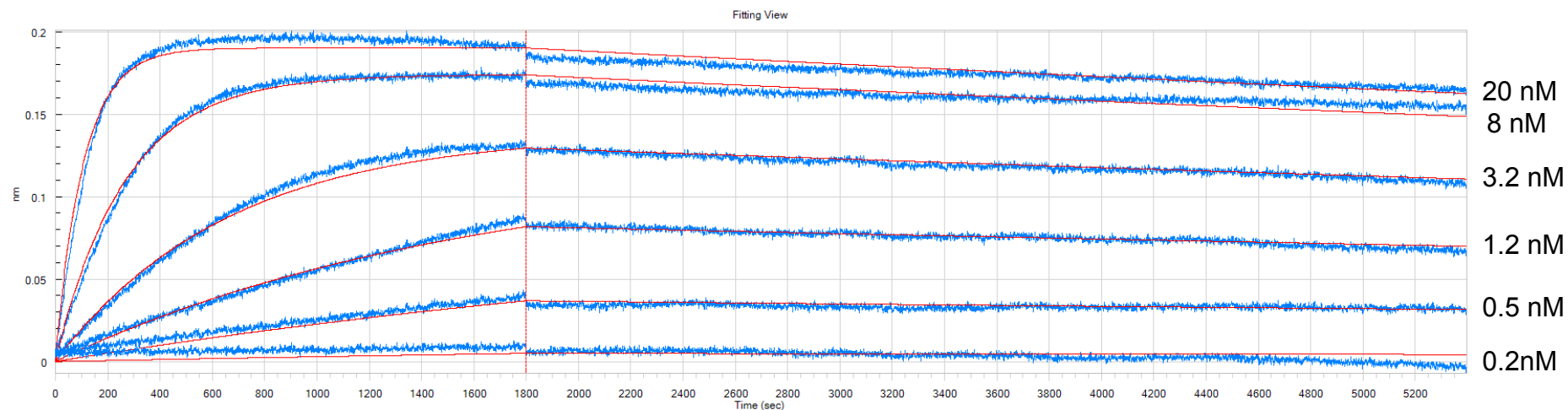
conc of Ag : 0.048 to 6.25 nM



K_D (nM)	K_{ass} ($M^{-1}s^{-1}$)	k_{diss} (s^{-1})
0.13	1.4×10^6	1.9×10^{-4}

K_D (nM)	K_{ass} ($M^{-1}s^{-1}$)	k_{diss} (s^{-1})
0.1	4.5×10^5	4.6×10^{-5}

Load Ab onto AHC biosensors, conc of Ag : 0.2 to 20 nM

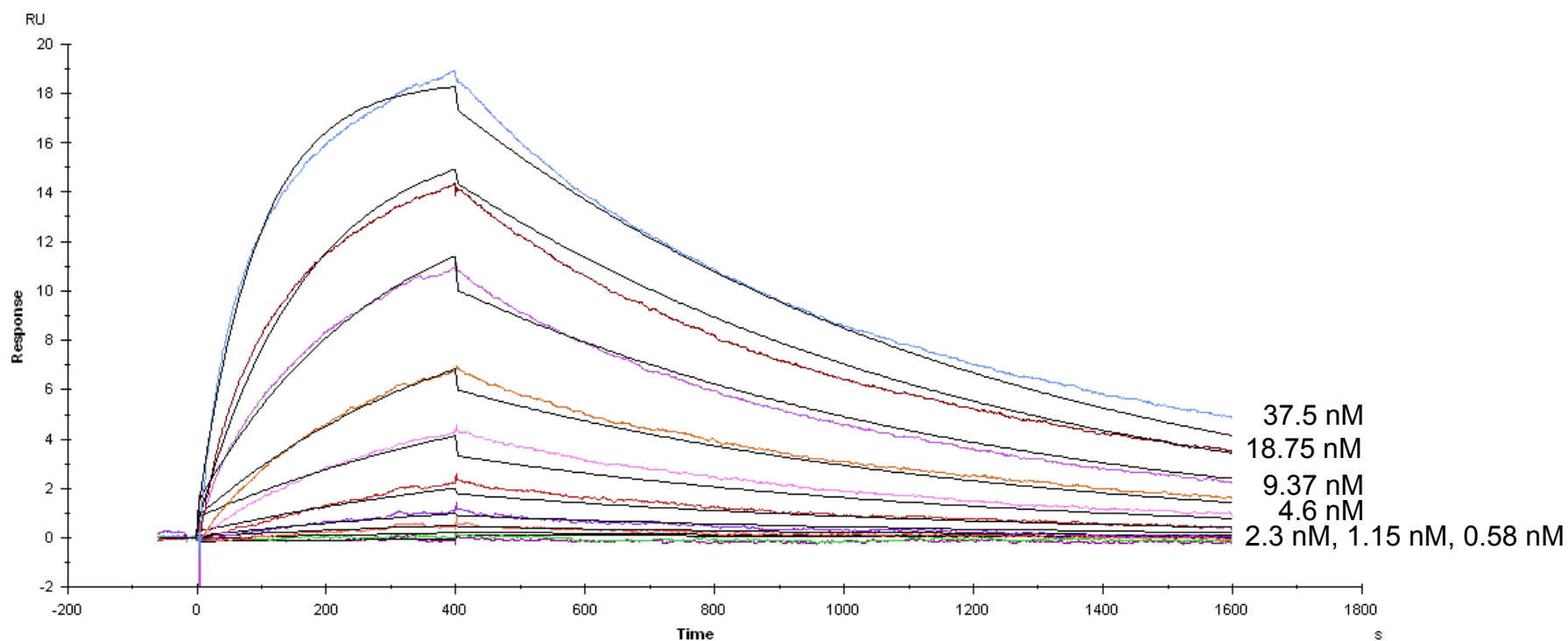




Kinetics constants for Example 2 (biacore/octetRed : different K_D)

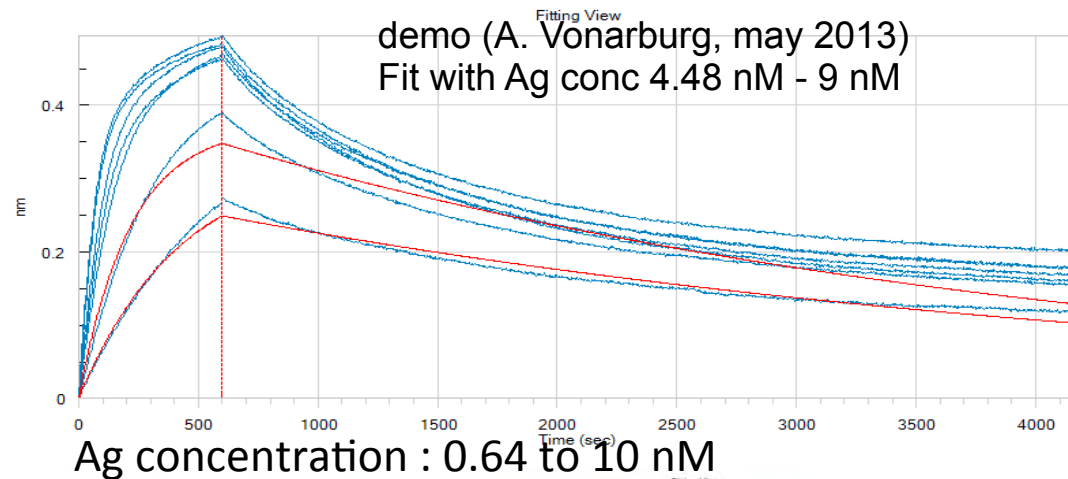
Ag concentration : 0.58 -37.5nM

(biacore T200)

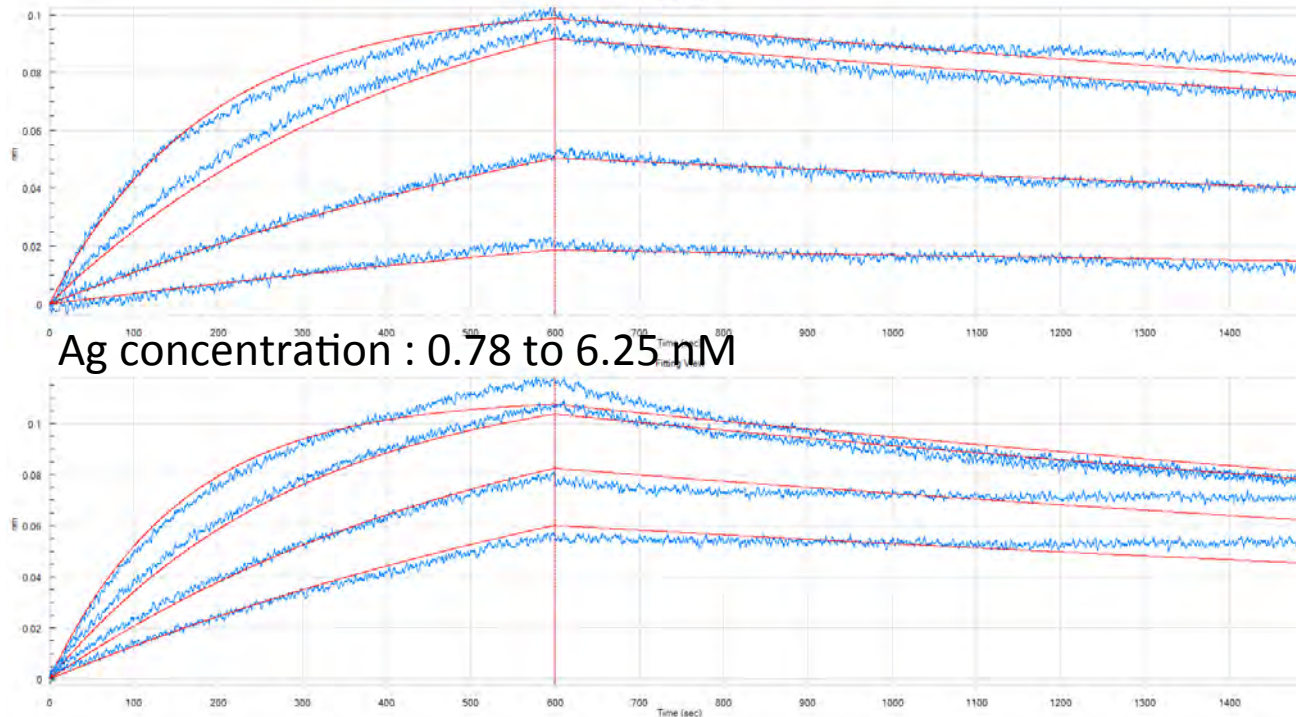


K_D (nM)	K_{ass} ($M^{-1}s^{-1}$)	k_{diss} (s^{-1})
4.7	2.54×10^5	1.2×10^{-3}

Kinetics constant for Example 2 (three different experiments with OctetRed)



Ab load onto streptavidin biosensors
exp 1 : about 2.2 nm
exp 2 and 3 : about 1.2 nm



K_D (nM)

0.57

0.46

0.37

Comparison of K_D constants from BiacoreT200 and OctetRed96

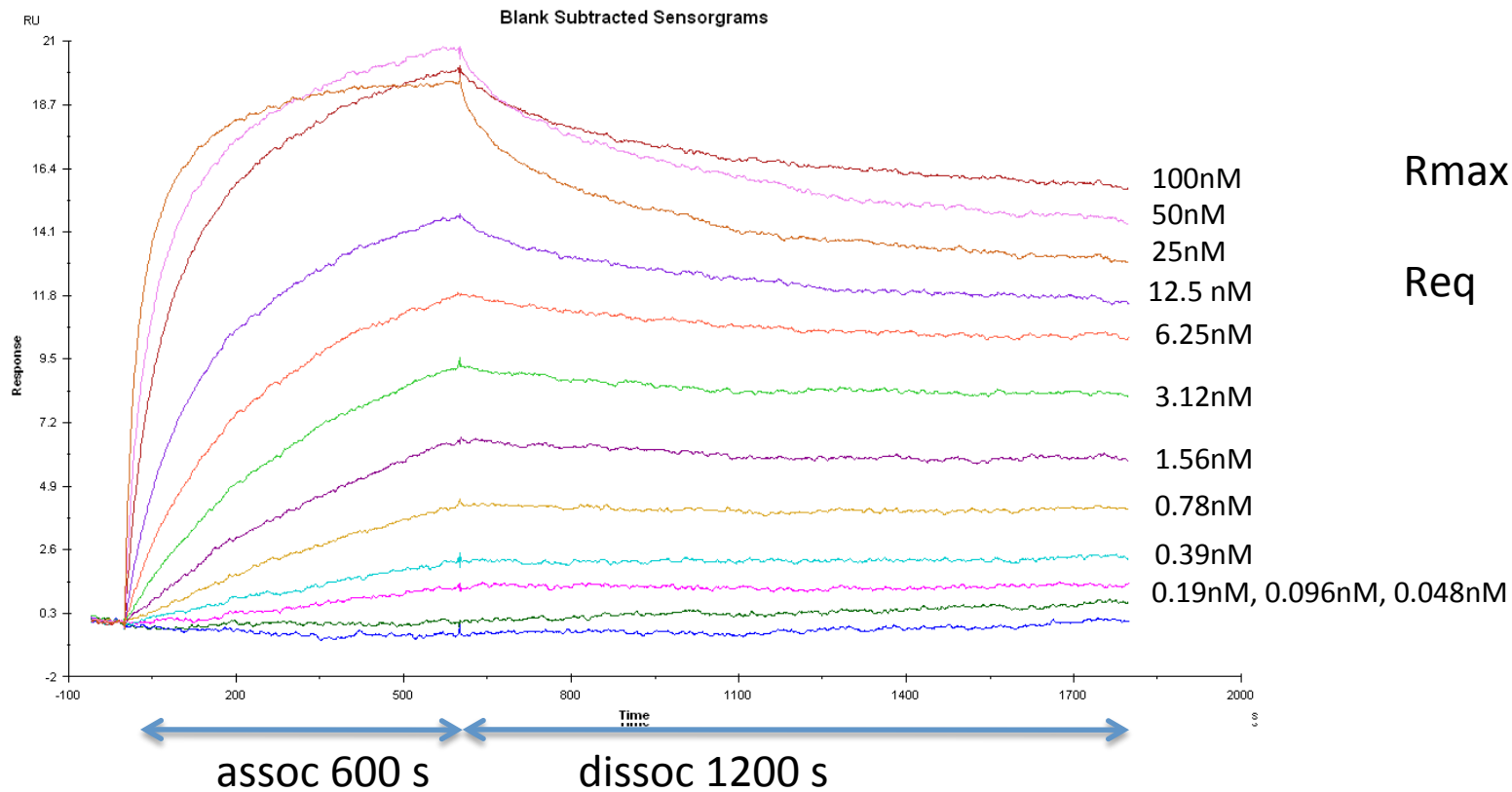
	KD (nM)	Kass ($M^{-1}s^{-1}$)	Kdiss (s^{-1})	
example 1	0.1	1.4×10^6	1.9×10^{-4}	biacore
	0.13	4.5×10^5	4.6×10^{-5}	octetRed
	0.76	3.11	4.13	biacore/octet
example 2	4.7	2.54×10^5	1.2×10^{-3}	biacore
	0.57	4.8×10^5	2.8×10^{-4}	OctetRed
	0.46	6.5×10^5	3.4×10^{-4}	OctetRed
	0.37	9.4×10^5	3.4×10^{-4}	OctetRed
	10.2	0.39	3.52	biacore/octet

lower with octet (x4)

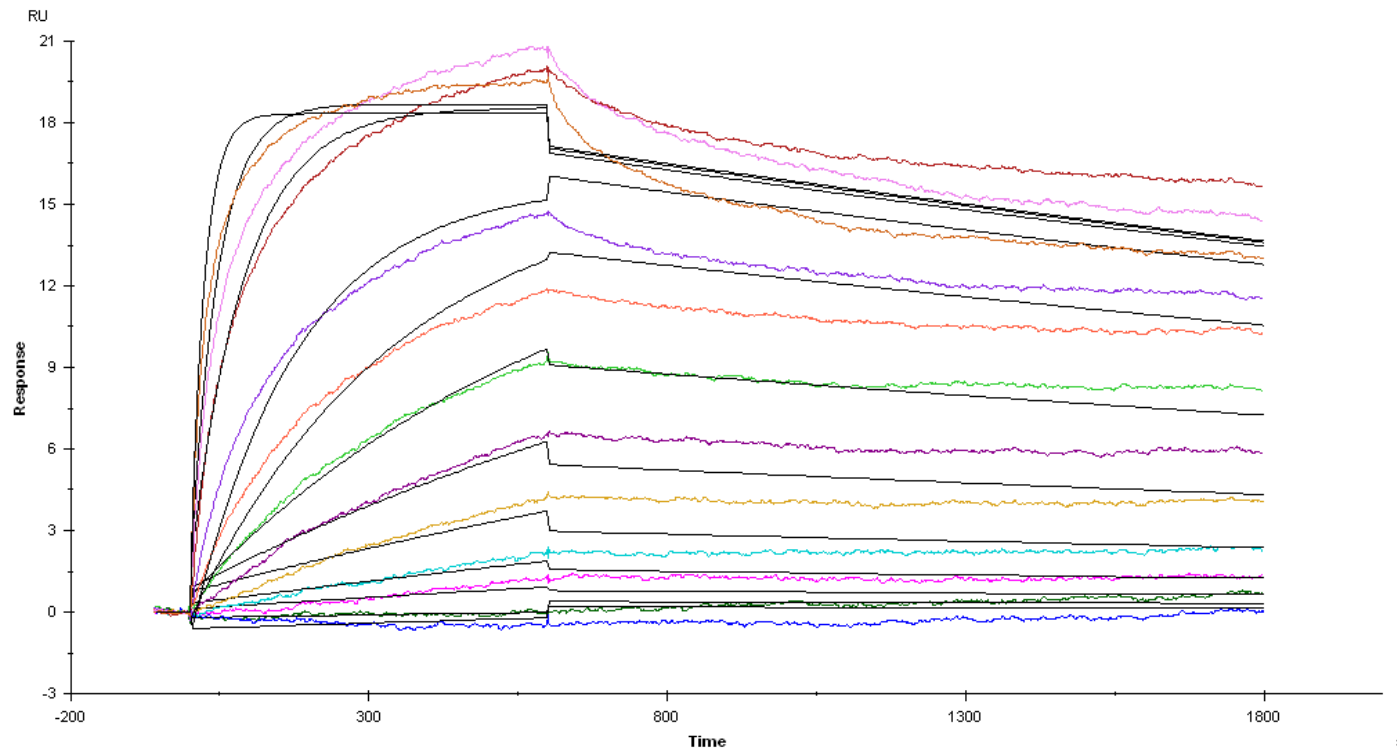
Parameters

- amount of ligand (immobilized/loaded)
- analyte concentration
- association time
- dissociation time

Example 3 : choice of analyte concentration for data fitting (Biacore T200)

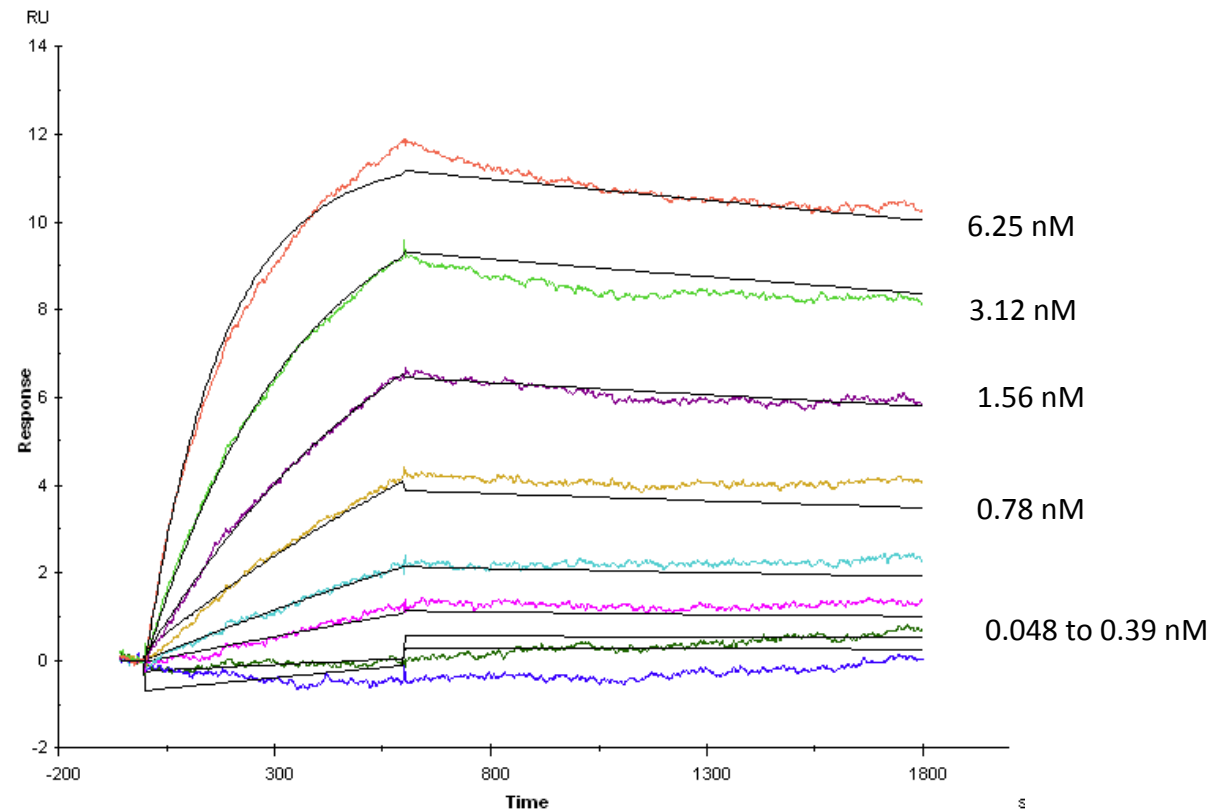


Example 3, fit of the data, model 1:1
with all concentrations (max : 100 nM)



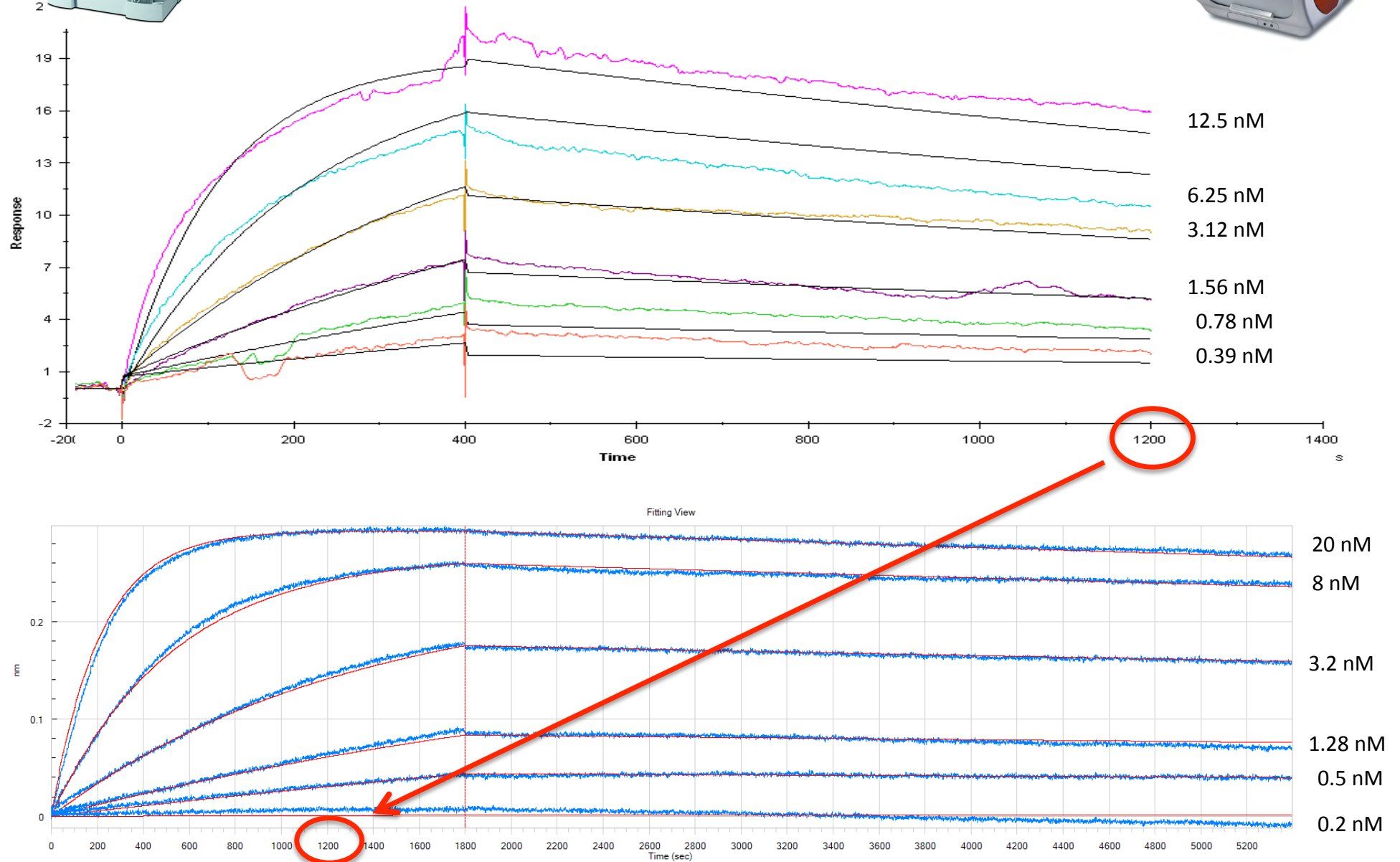
Bad fitting, $K_D = 0.43$ nM

Example 3, fitting of the data, model 1:1
with concentrations « in the range » of the KD

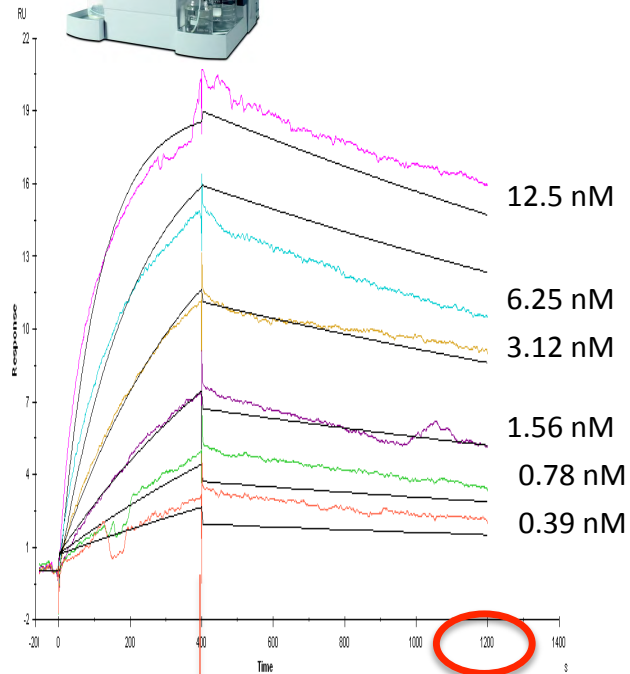


K_D (nM)	$K_{ass}(M^{-1}s^{-1})$	$K_{diss}(s^{-1})$
0.1	8.8×10^5	9×10^{-5}

Example 4 : different dissociation constants using biacore and OctetRed



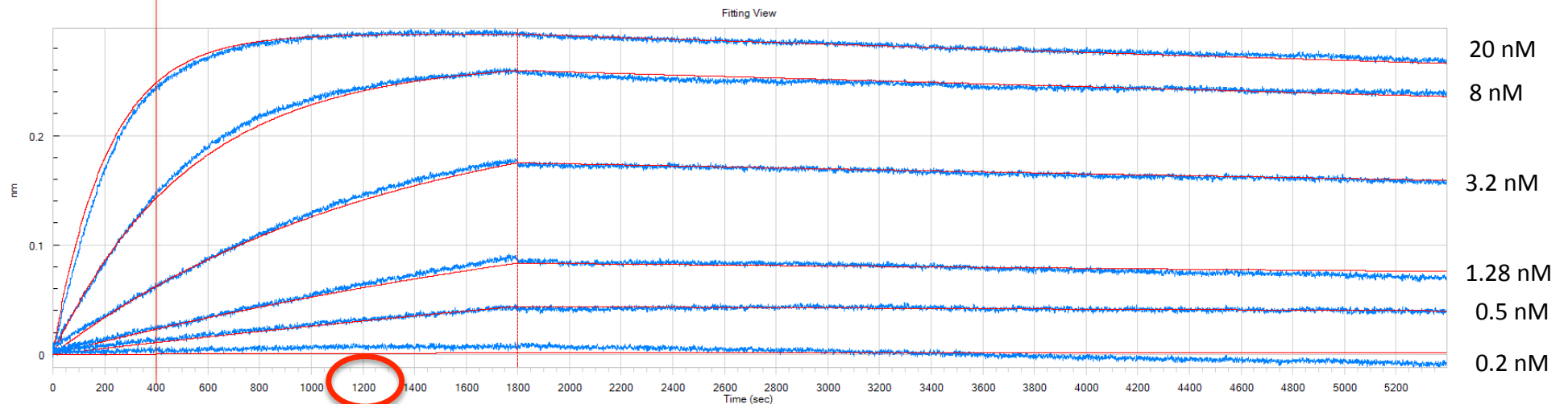
Example 4 : different dissociation constants using biacore and OctetRed



KD (nM)	K _{ass} (M ⁻¹ s ⁻¹)	K _{diss} (s ⁻¹)	
0.44	7×10^5	3×10^{-4}	biacore
0.11	2.33×10^5	2×10^{-5}	octetRed

Total time [Biacore = $6 \times (1200 + 600) = 10800$ s
OctetRed = 5400 s

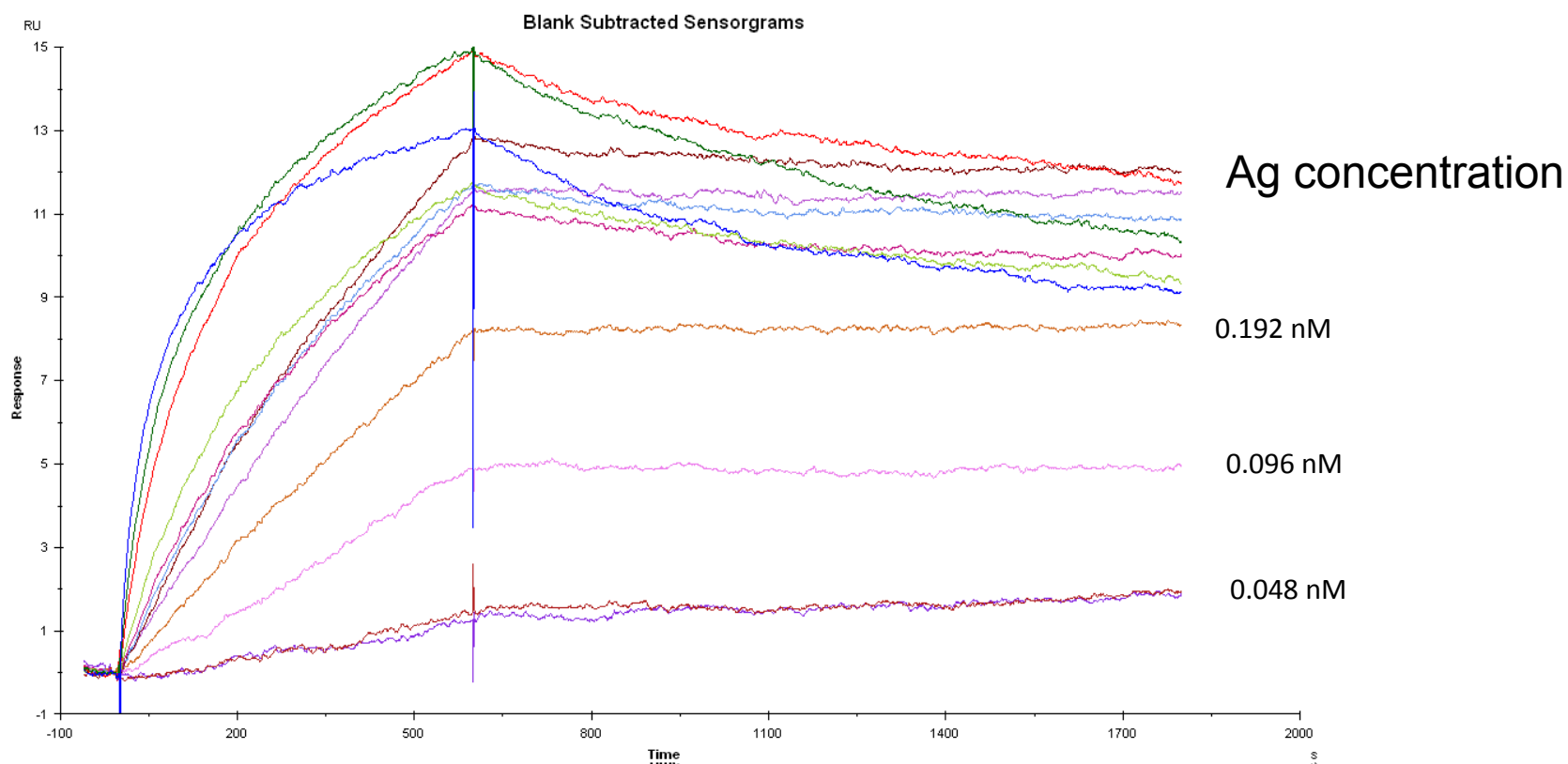
for equivalent experiment : $6 \times (5400 + 600) = 36000$ s





Example 5 : very tight complex (K_D around 10^{-12} M)

Biacore T200

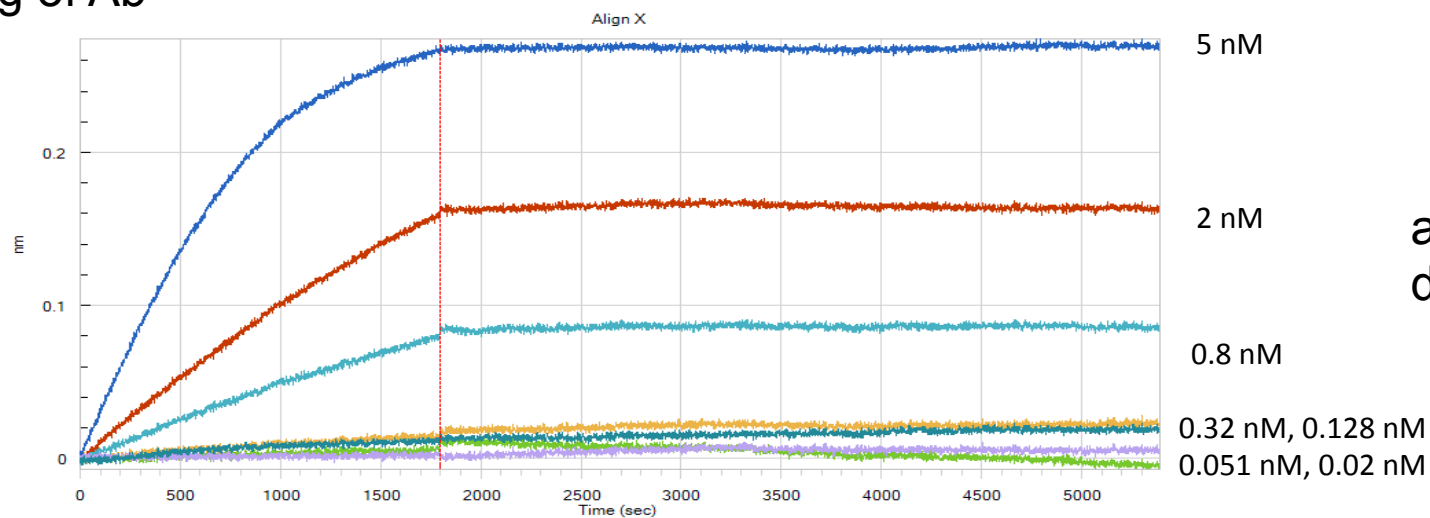


Ab immobilized on Fc4 (Fc1=reference, Fc2 and Fc3 = 2 Ab => OK)

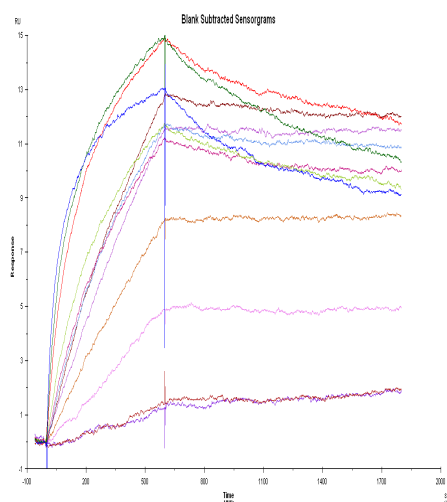
NO DATA FIT POSSIBLE
(needs for protocol optimization)

Example 5 : kinetic experiment on AHC biosensors using octetRed96

loading of Ab



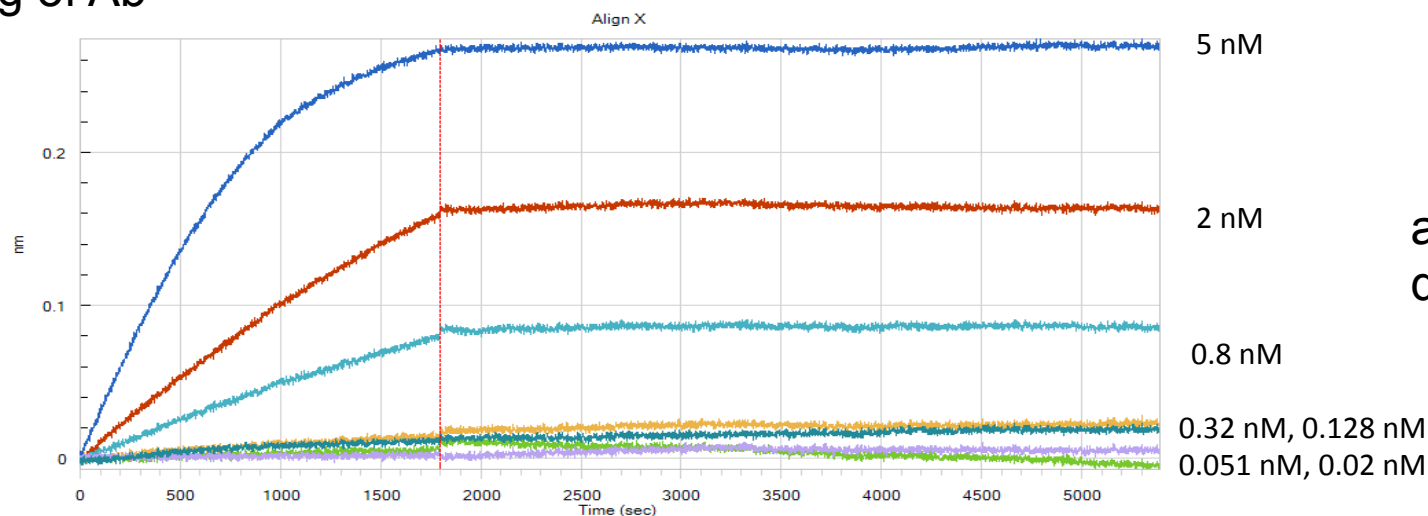
assoc 1800s
dissoc 3600s



Example 5 : kinetic experiment on AHC biosensors using octetRed96

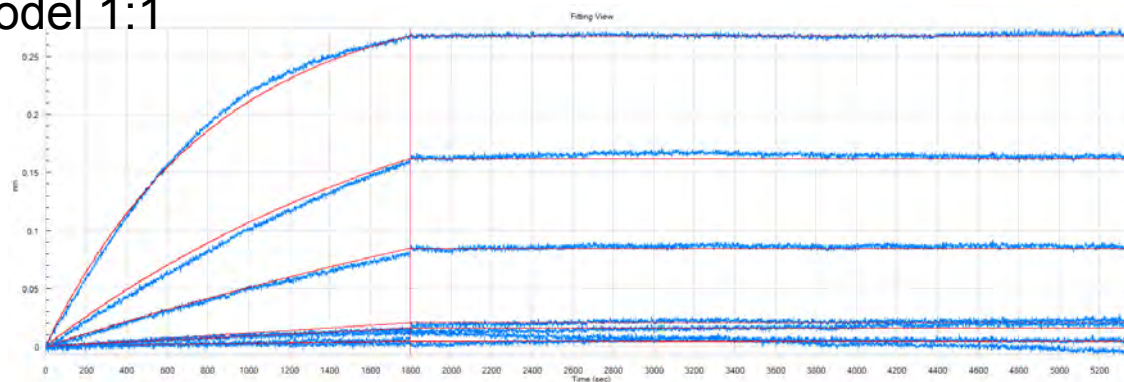


loading of Ab



assoc 1800s
dissoc 3600s

Fit model 1:1



increase
dissociation
time ?

KD (nM)	Kass(M ⁻¹ s ⁻¹)	Kdiss(s ⁻¹)
< 0.001	2.35 x 10 ⁵	< 10 ⁻⁷

Octet RED96
10 ² – 10 ⁷
10 ⁻⁶ – 10 ⁻¹
1 mM – 10 pM
10 ² – 10 ⁷
10 ⁻⁶ – 10 ⁻¹
1 mM – 10 pM

C'est fini !

