RéNaFoBiS

afmb
architecture et fonction
des macromolécules biologiques

Aix*Marseille
université

Approches complémentaires pour l'analyse des interactions moléculaires.

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Ecole Nationale de Biologie Structurale Intégrative

Juin 2015
Ile d'Oléron

How to characterize a macromolecular complex?

Stoichiometry

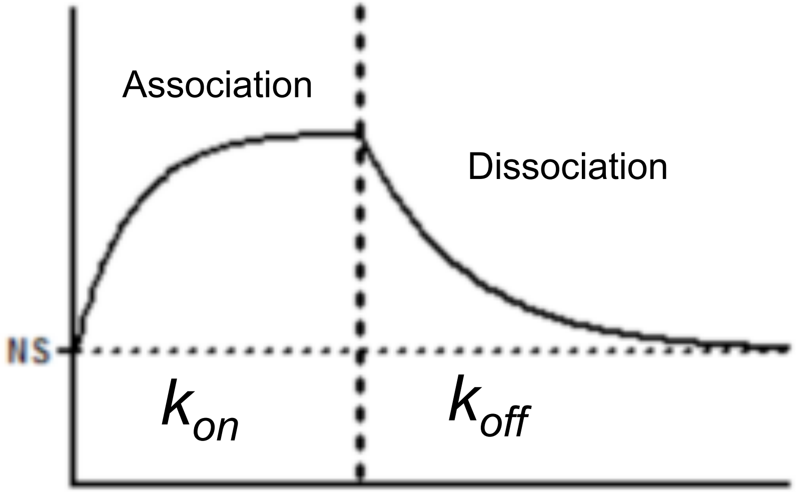
Stability

Affinity

Kinetics

Thermodynamic parameters

Binding kinetics

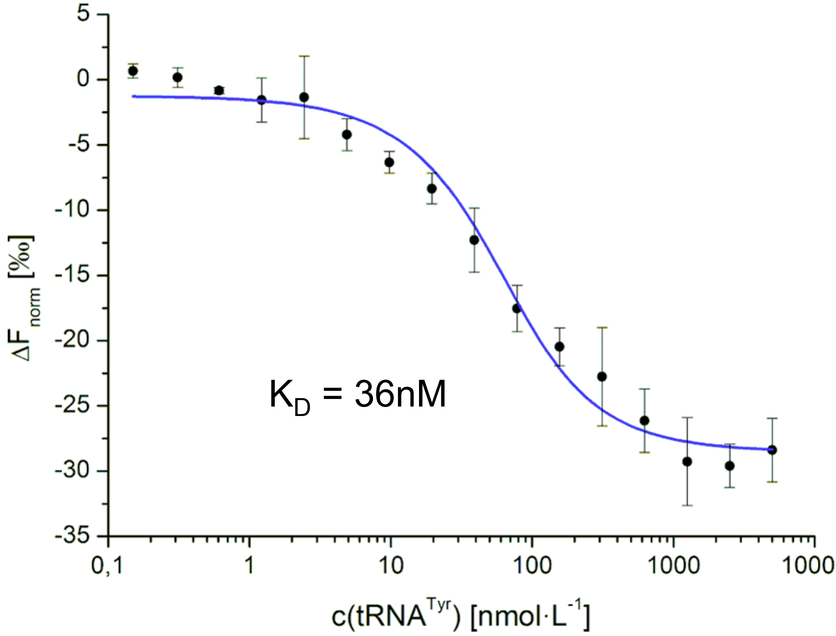


Association: how fast molecules bind = $M^{-1}s^{-1}$

$$A + B \underset{k_{off}}{\overset{k_{on}}{\rightleftarrows}} AB$$

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

Equilibrium



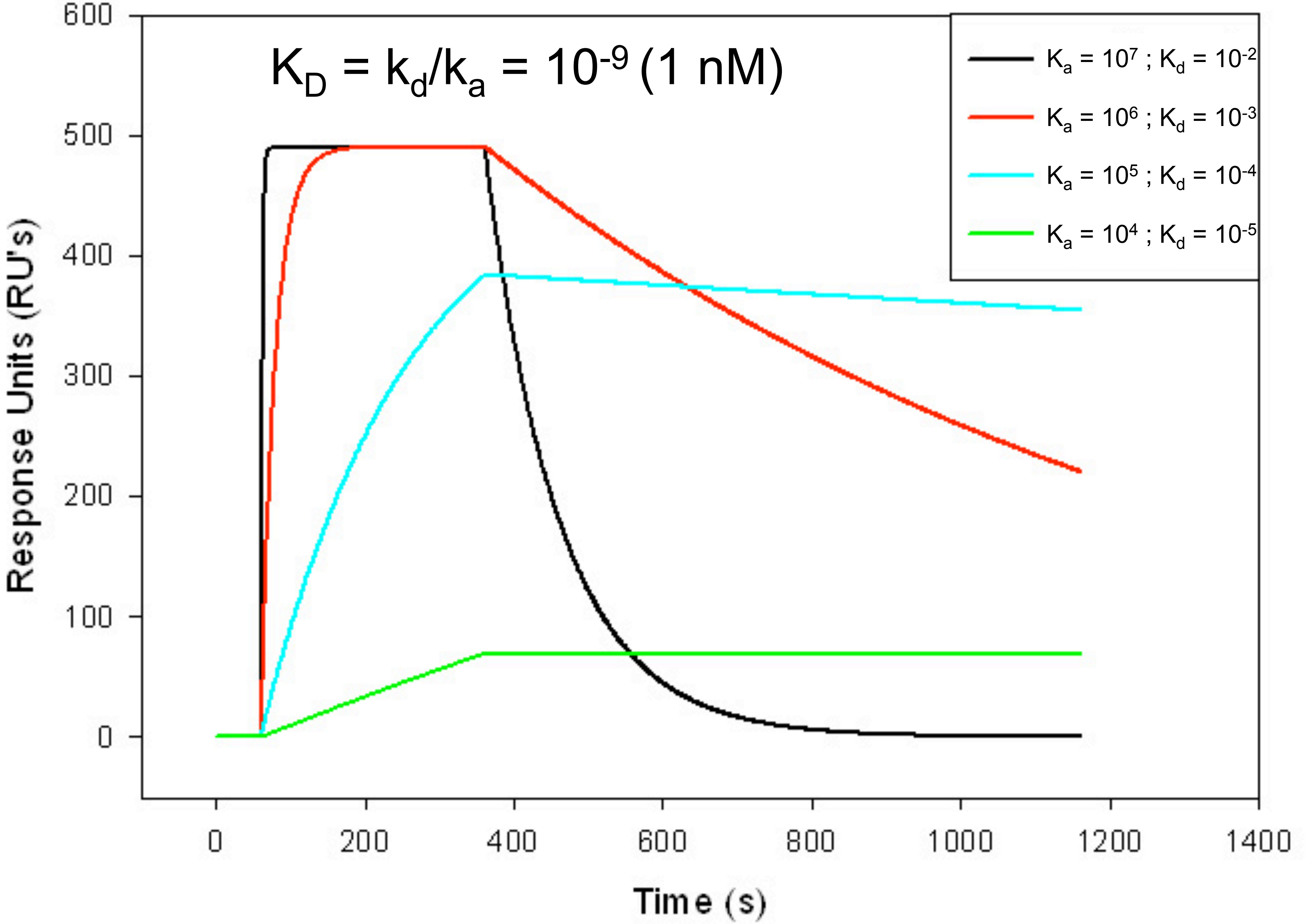
forward binding = backward unbinding

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

Equilibrium dissociation constant

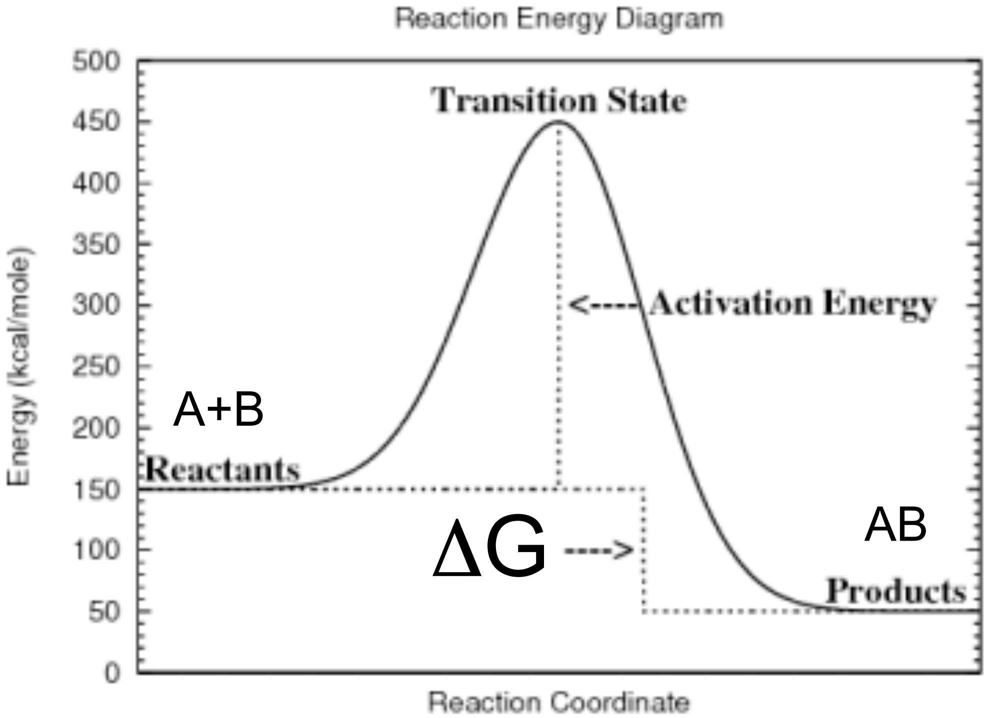
$$K_D = [A][B] / [AB] = k_{off} / k_{on}$$

Same Affinity … Different Kinetics



$K_D = k_d/k_a = 10^{-9}$ (1 nM)

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)

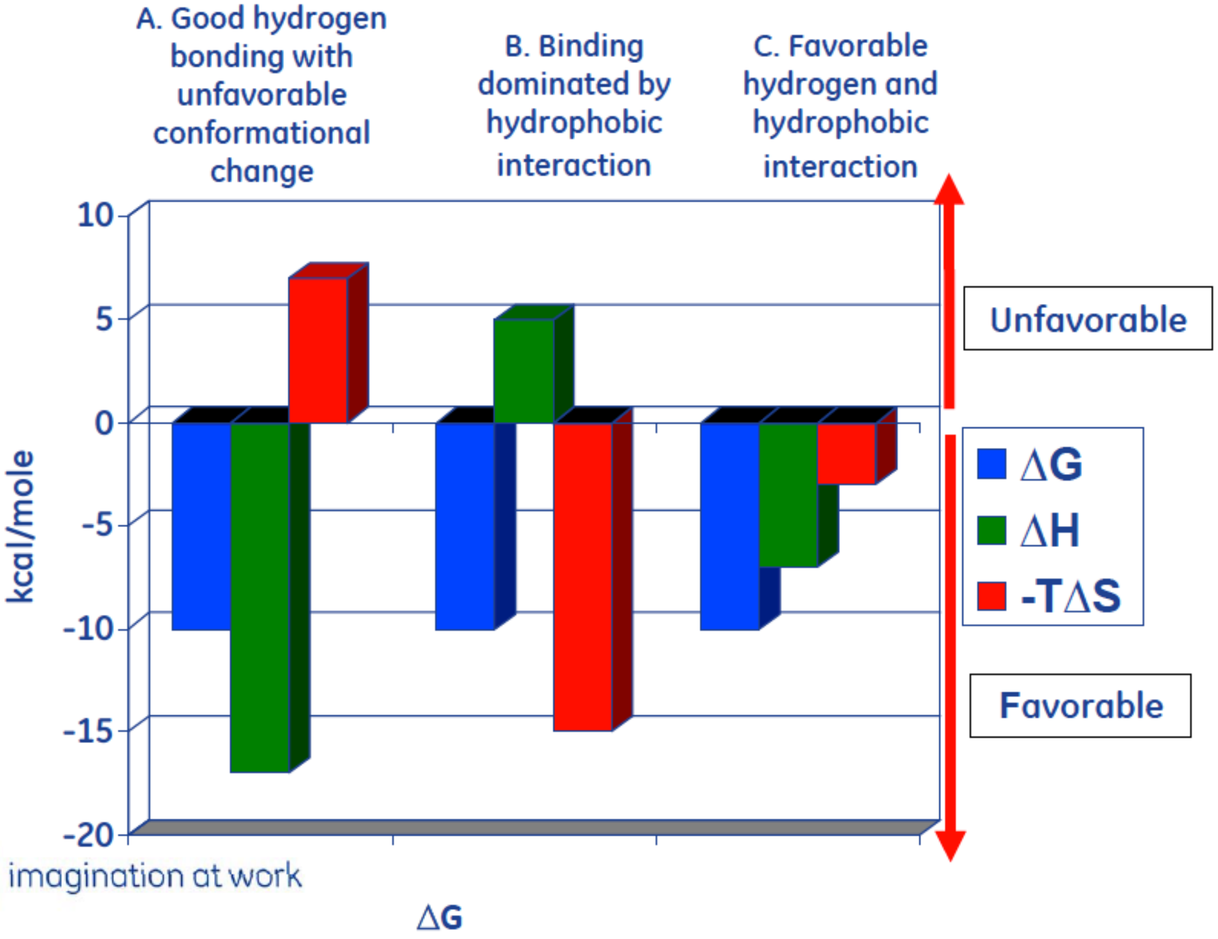
$$\Delta G = RT\ \ln(K_D)$$

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

Affinity is just part of the picture

MicroCal
Part of GE Healthcare

All three interactions have the same binding energy (ΔG)



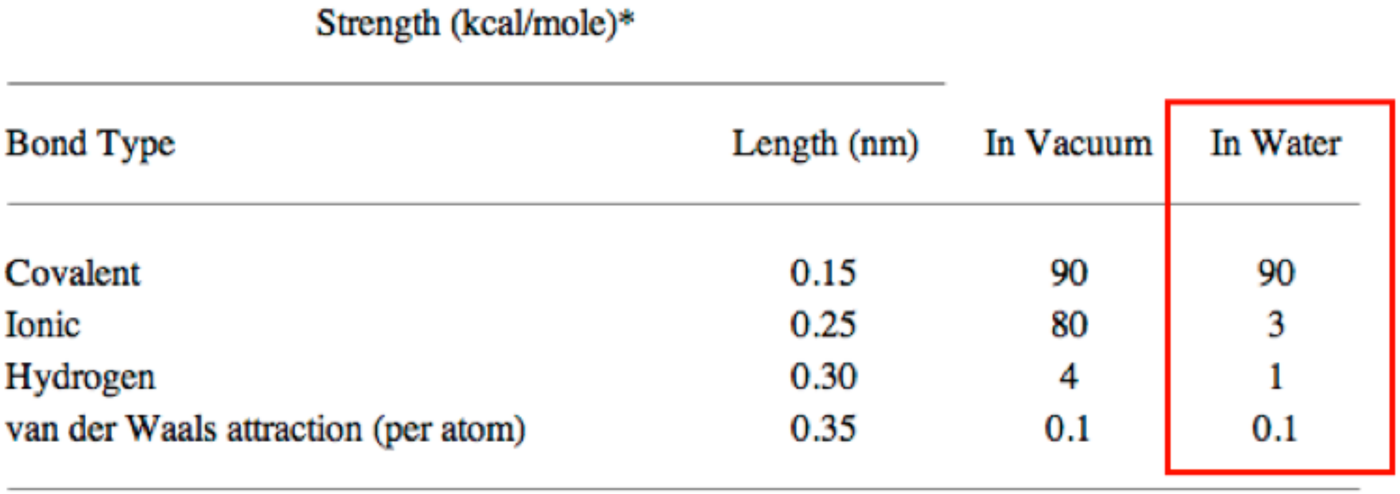
imagination at work

Binding equilibria and free energy

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

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



Bond Type	Length (nm)	Strength (kcal/mole)* In Vacuum	Strength (kcal/mole)* 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

Molecular Biology of the Cell (NCBI bookshelf)

AFMB
Architecture et Fonction
des Macromolécules Biologiques

FRISBI
Integrated Structural Biology

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…

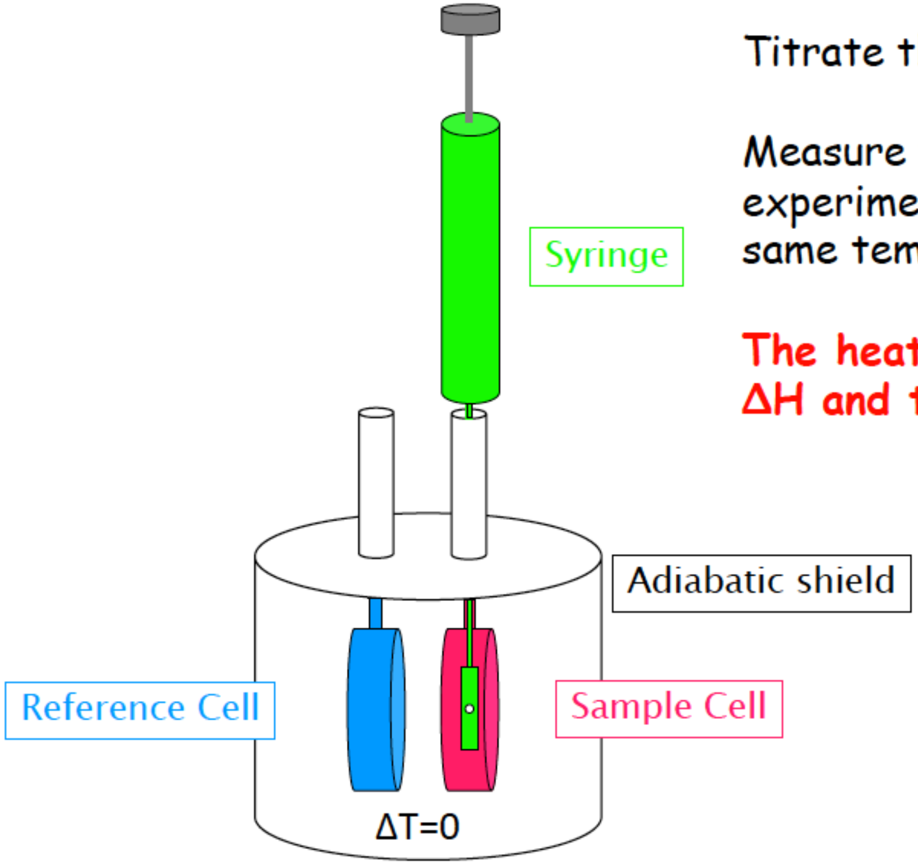
GE Healthcare

ITC

Isothermal
Titration
Calorimetry

MicroCal ITC 200

What happens during an ITC experiment?



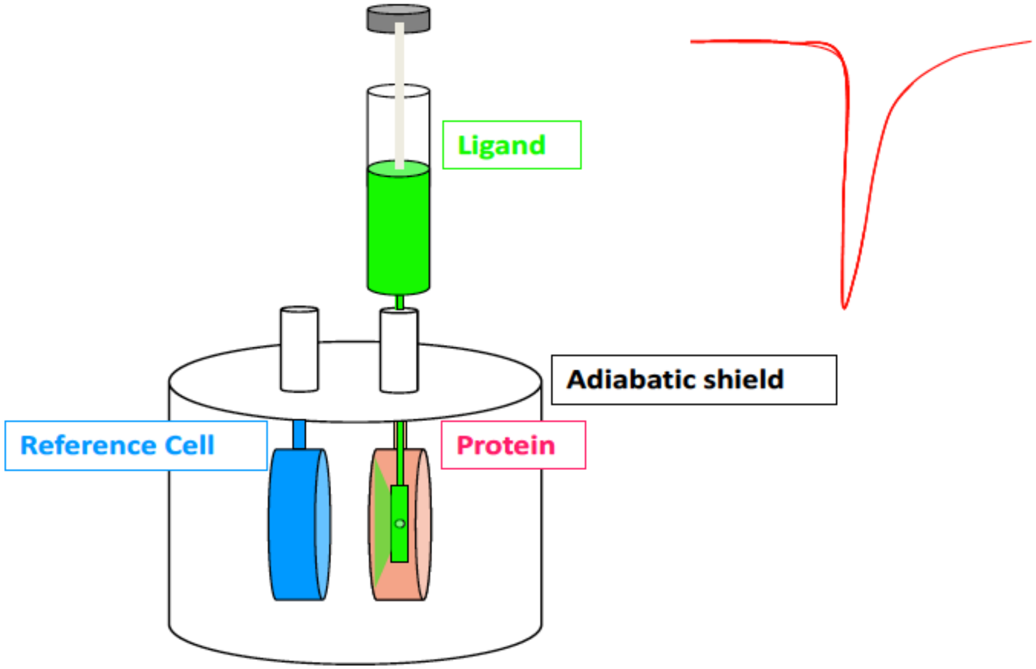
Protein target in sample cell

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

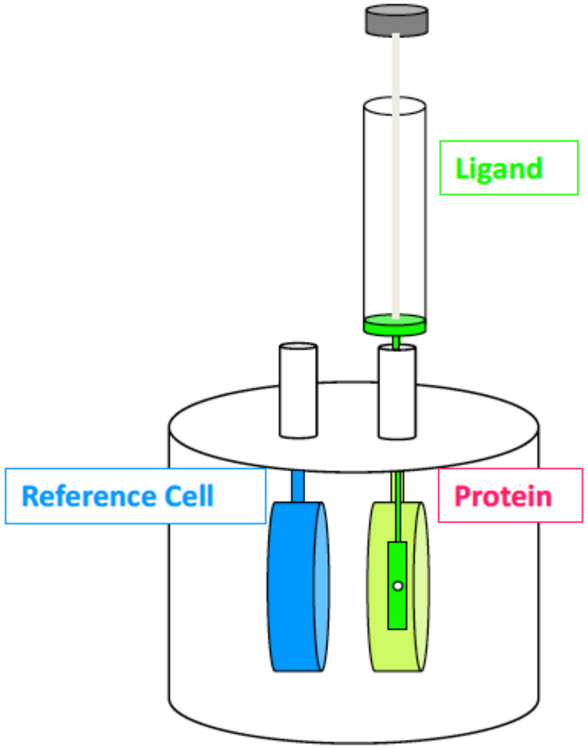
➔ Heat absorbed in sample cell

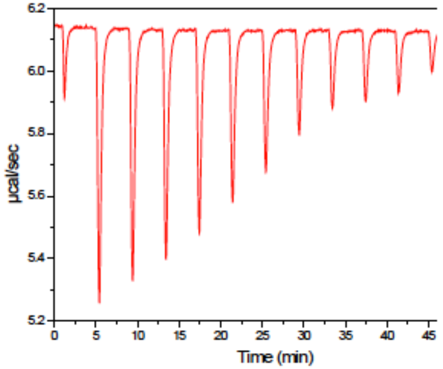
➔ extra energy will be provided in order to keep $\Delta T=0$

Exothermique reaction

➔ Heat released in sample cell

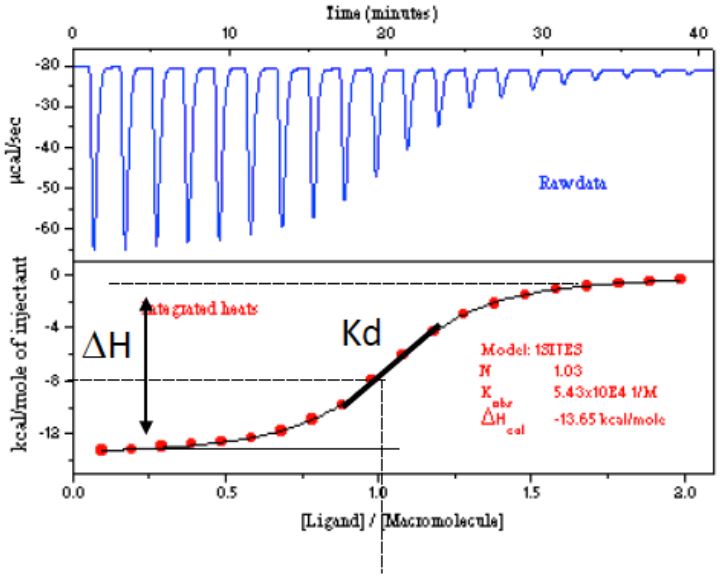
➔ less energy will be provided in order to keep $\Delta T=0$





- 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]



Stoichiometry (n)

Data points are fitted according to binding models

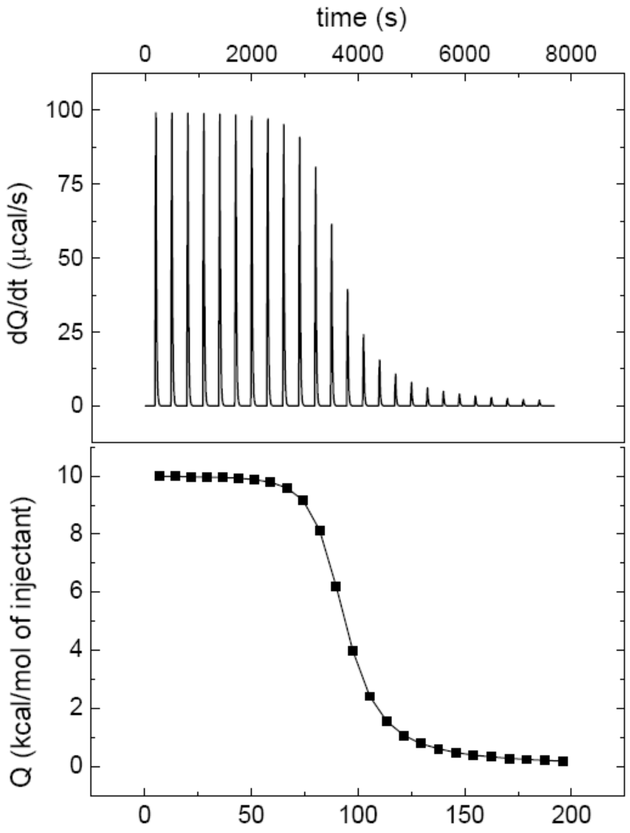
ΔH, n and K_D are calculated from the fitting

ΔG and ΔS are derived from the equations:

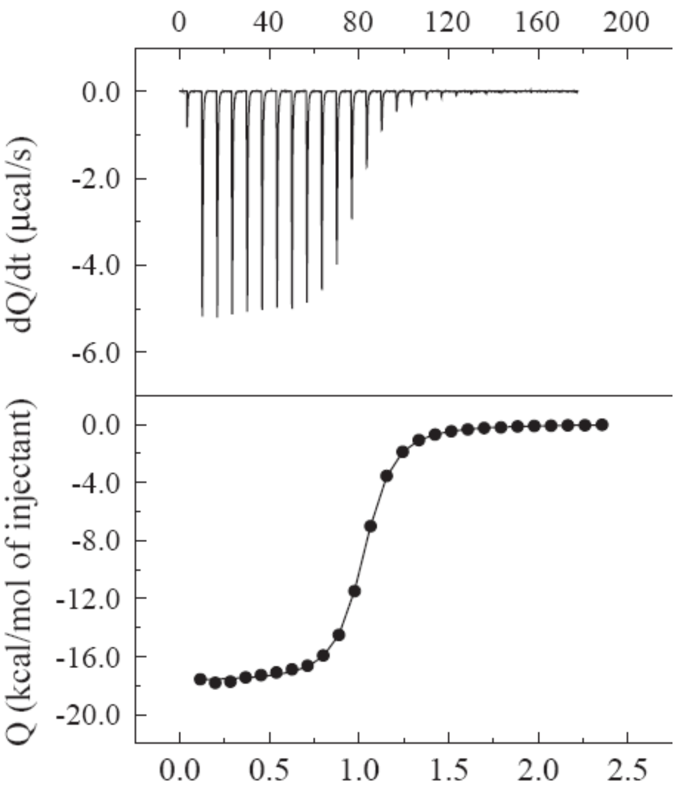
$$\Delta G = RT \ln K_D$$

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

Endothermique ($\Delta H>0$)



Exothermique ($\Delta H<0$)



MST

Micro Scale Thermophoresis

Monolith NT.115

What is Thermophoresis?

Electrophoresis:

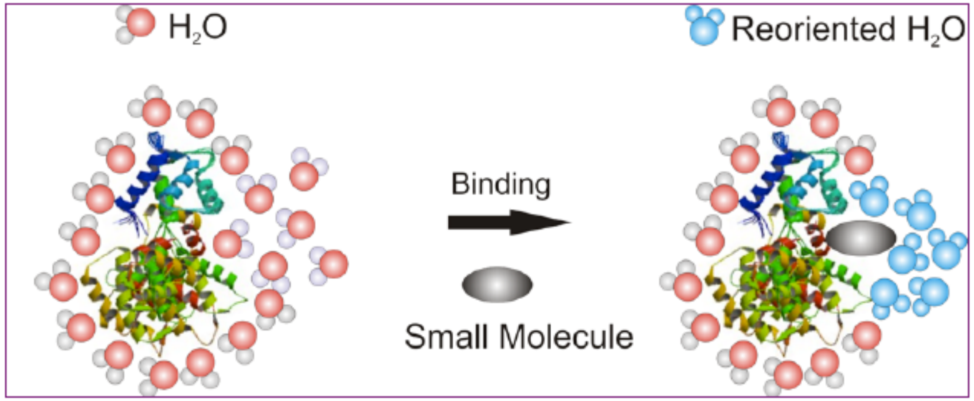
We apply an electric field
We separate the molecules by charge (and also size)

Thermophoresis:

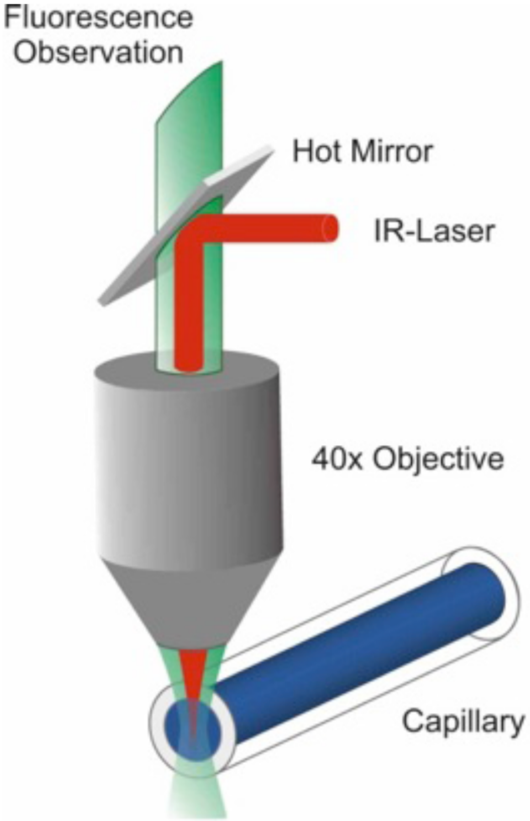
Sample is in solution in a capillary
An infrared laser generates a temperature gradient (between 1-6 K)
Thermophoresis is the motion of molecules in temperature gradients.

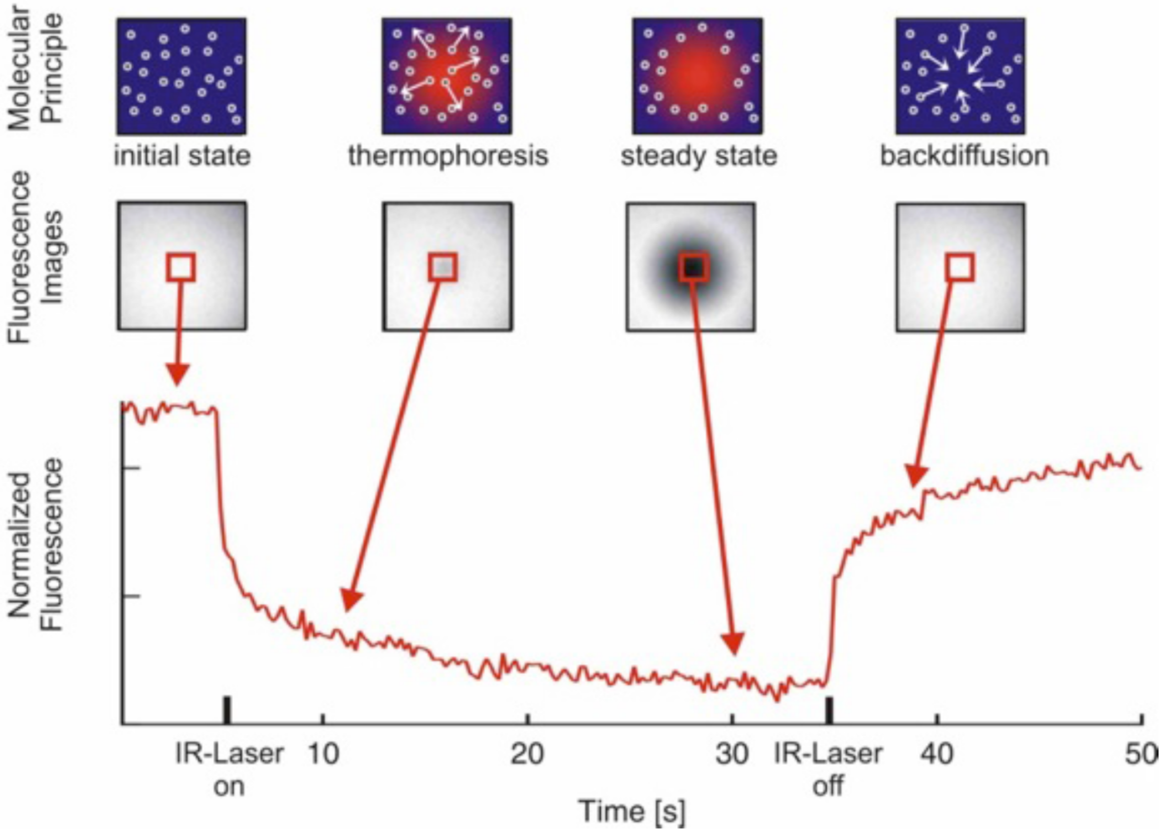
Movement is detected by through fluorescence of one of the binding partners.

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



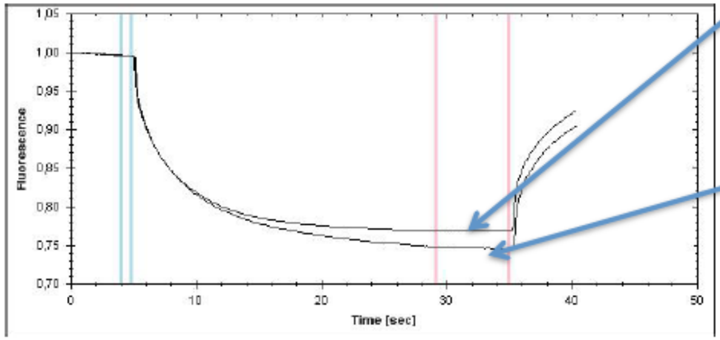
Thermophoresis experiment





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

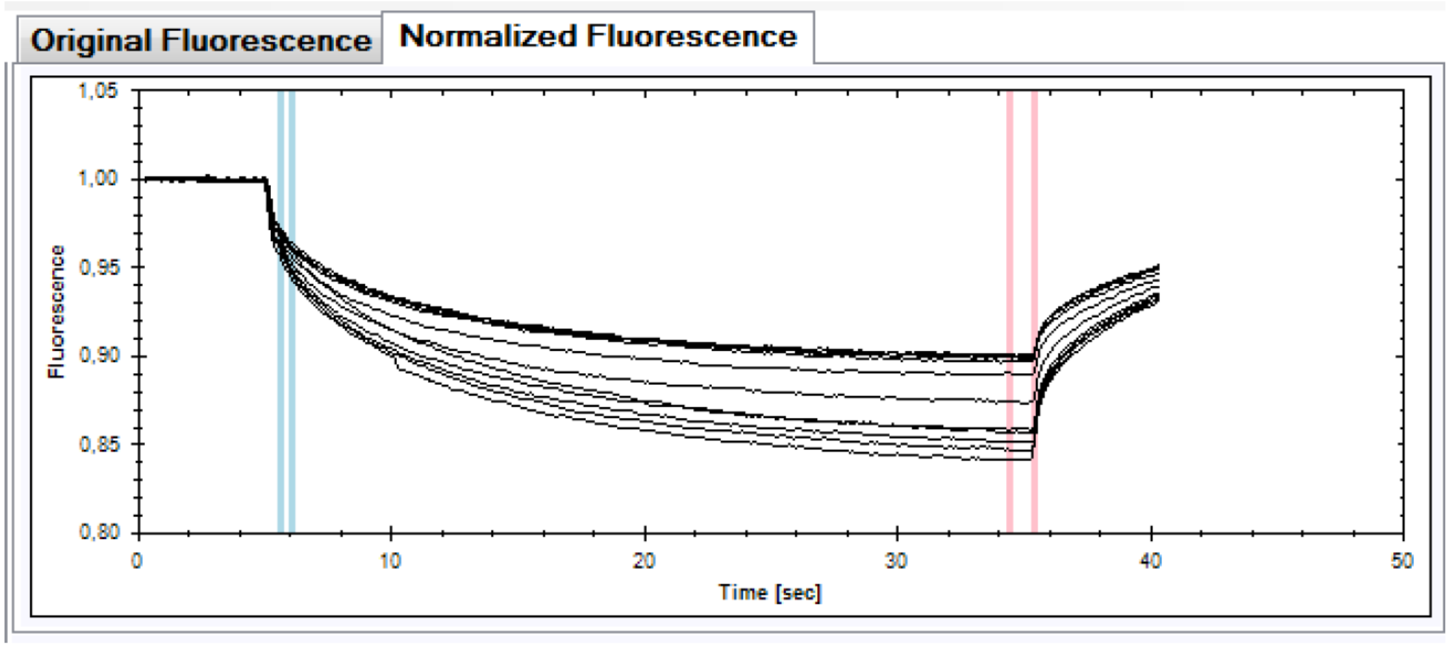
Thermophoresis mesurement (MST) on first capillary (highest concentration of ligand)



MST on second capillary

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

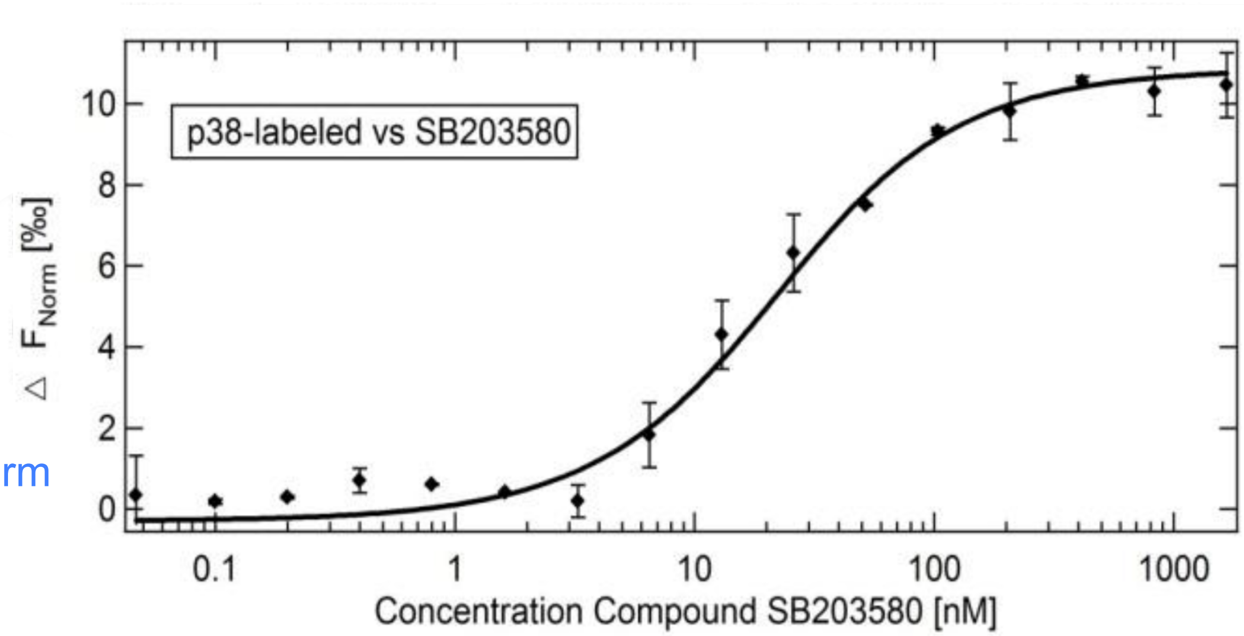
Thermophoresis will depend on size + charge + hydration shell + conformation



Measurement
over 16
capillaries

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

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



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)

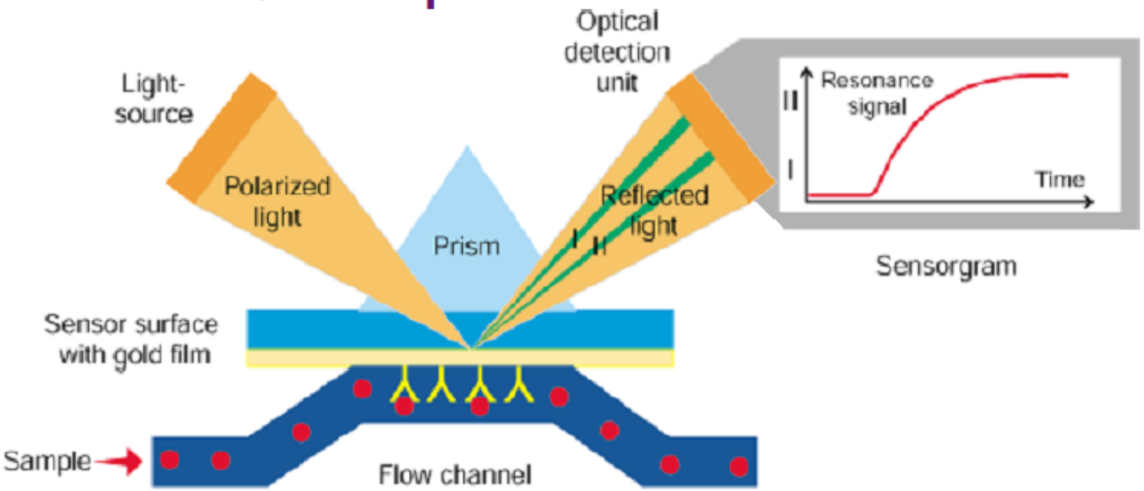
GE Healthcare

SPR

Surface
Plasmon
Resonance

Biacore T200

Surface plasmon resonance



Physical phenomenon: Surface plasmon resonance

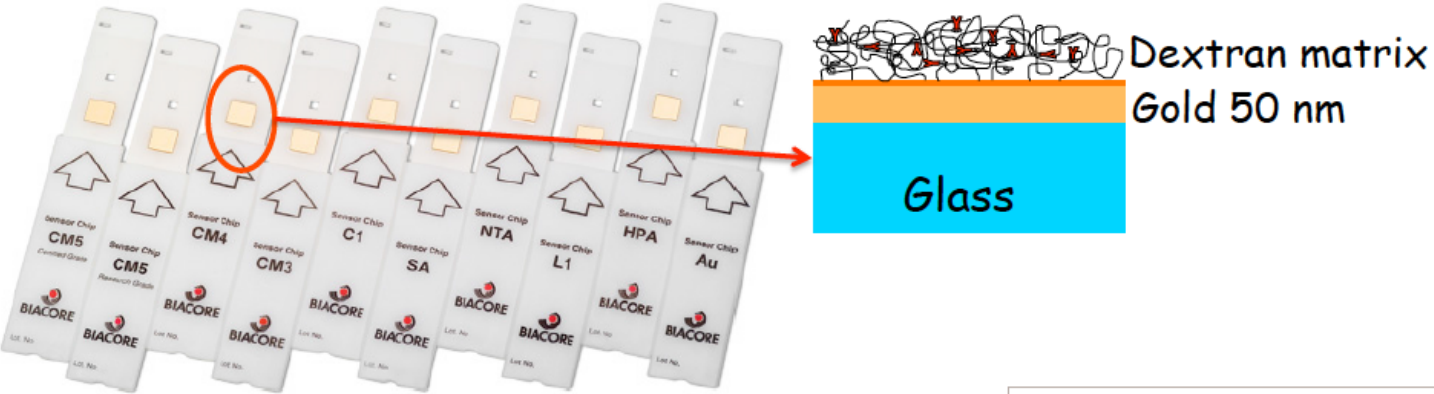
SPR machine detects refractive index changes close to the sensor surface

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

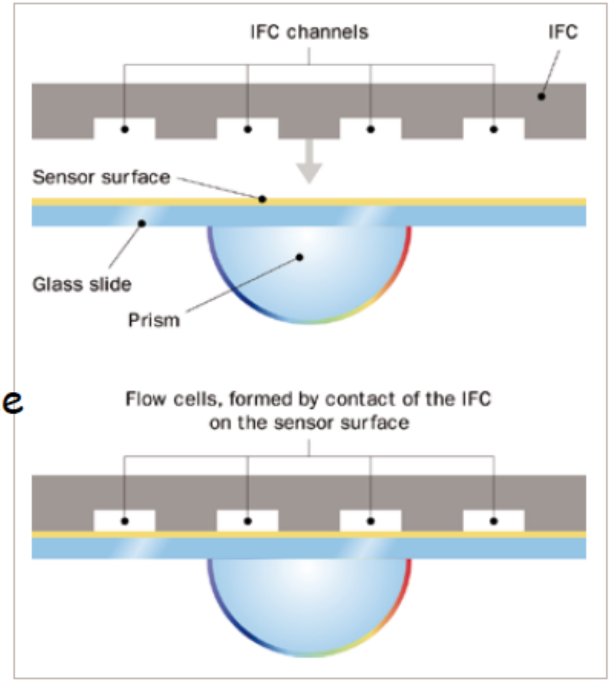
Binding of the analyte will change the refractive index near the surface, this change will generate a resonance signal that will be measured in real time and represented in a sensorgram

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

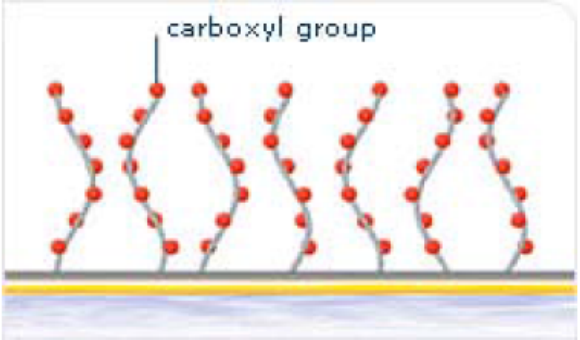


Flow cells are formed by pressing an integrated microfluidic cartridge (IFC) against a sensor surface

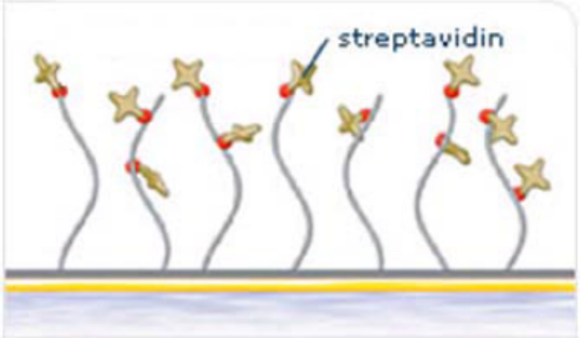


Different types of sensor chips

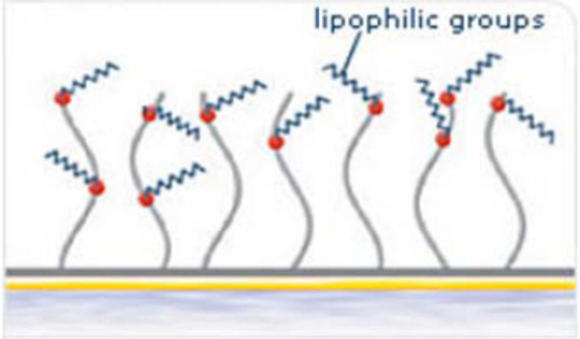
CM5 (carboxymethyl-dextran) Coupling via -NH2, -SH, -CHO, -OH or -COOH



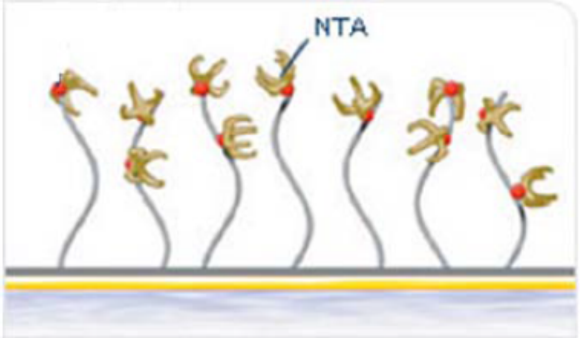
SA (immobilization of biotylilated molecules)



L1 (immobilization of membrane structures)

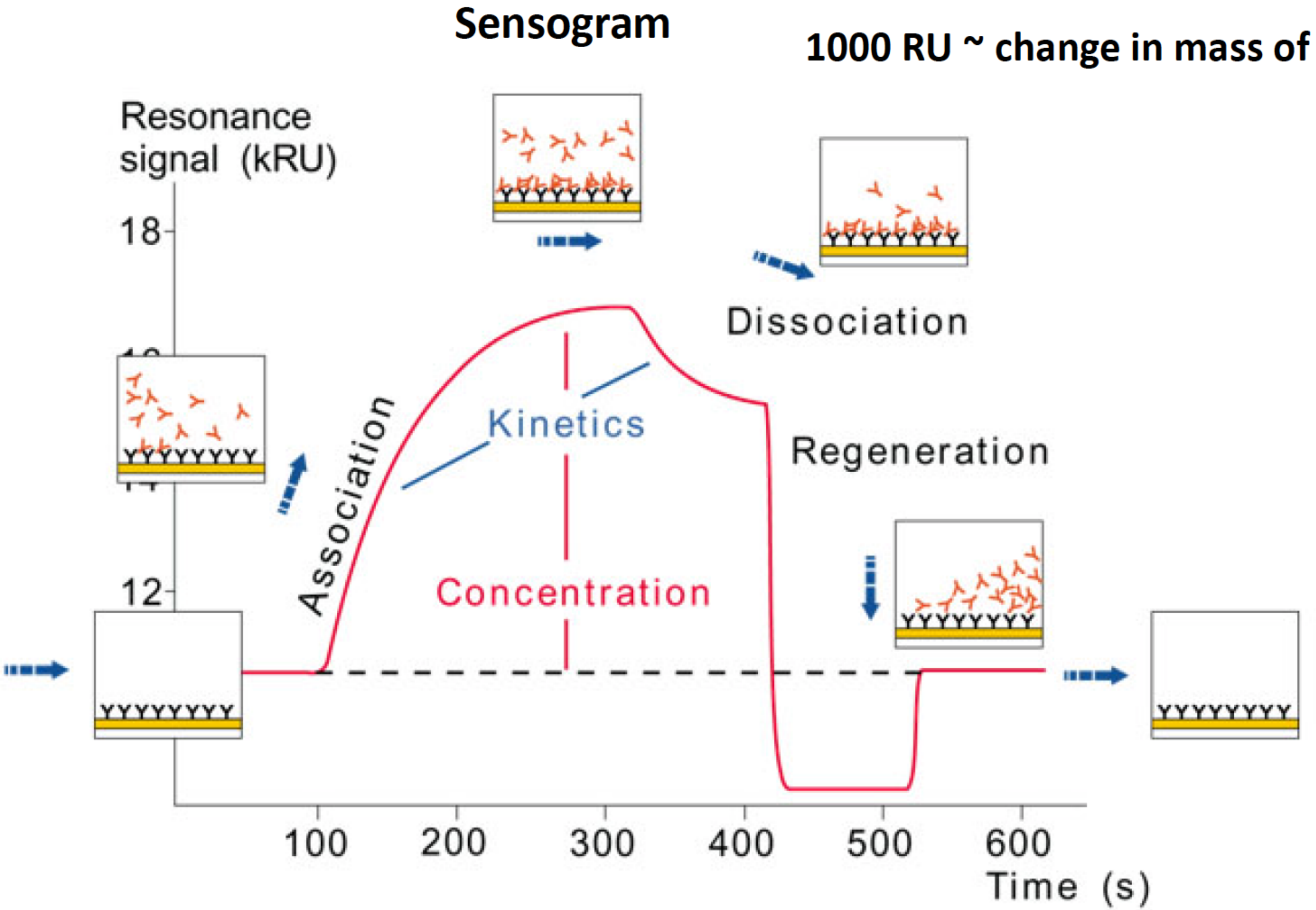


NTA (binding of Ni^{2+} / his-tagged proteins)

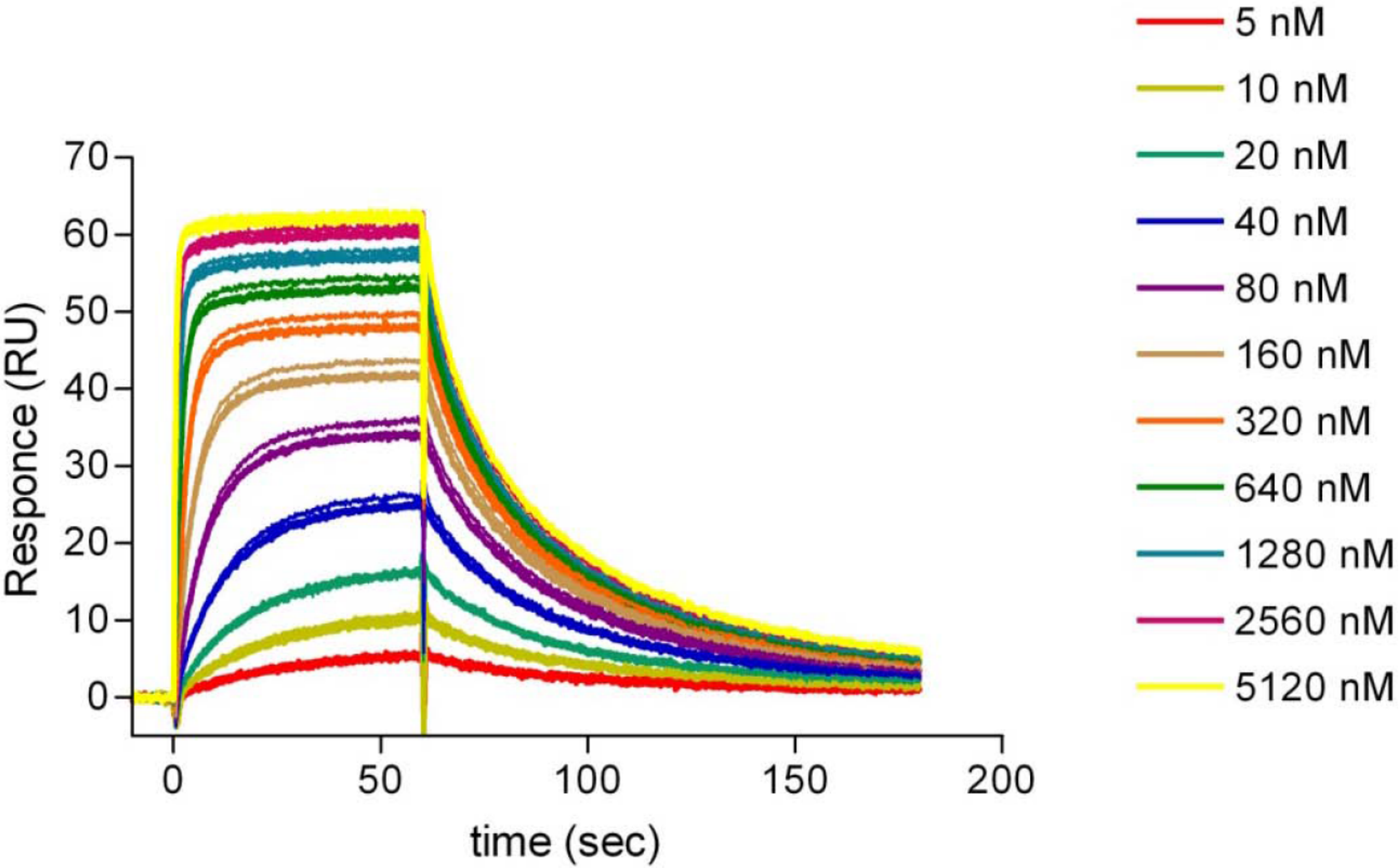


Typical binding kinetic's experiment

1000 RU ~ change in mass of 1ng/mm^2



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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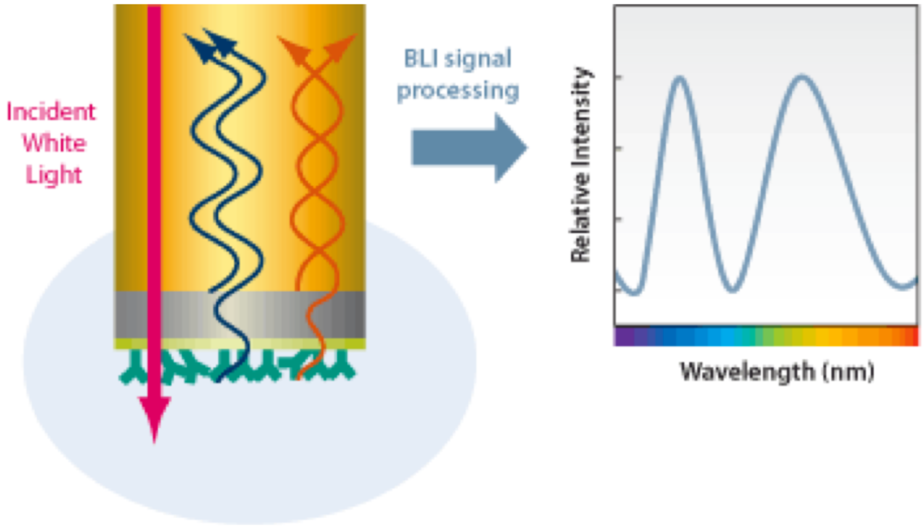
A Division of **Pall Life Sciences**

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

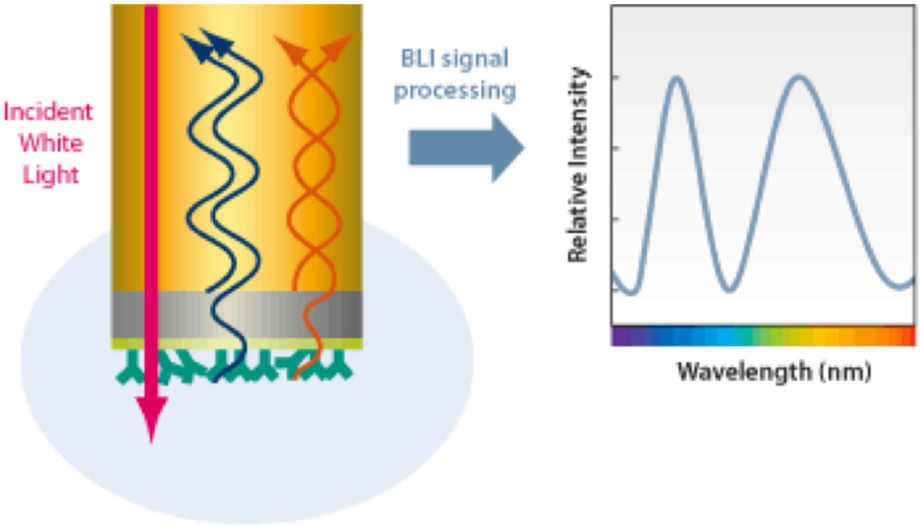
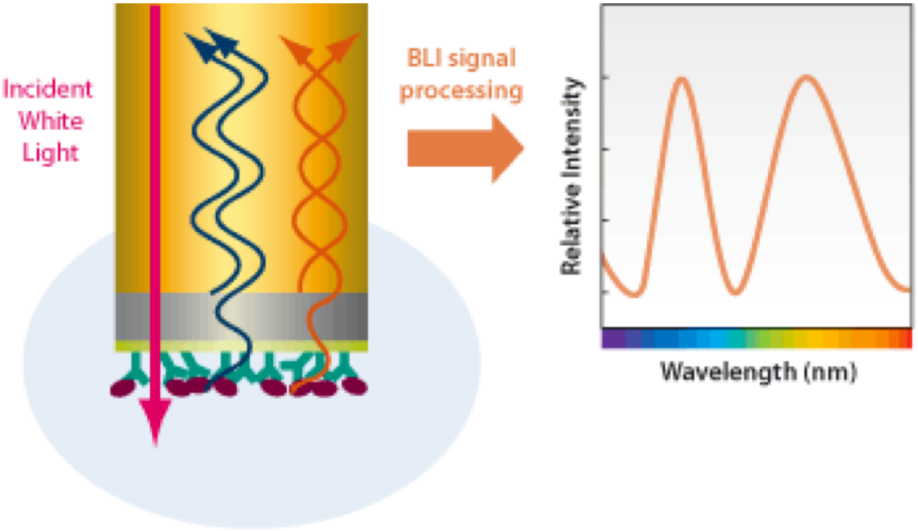


Figure 1

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

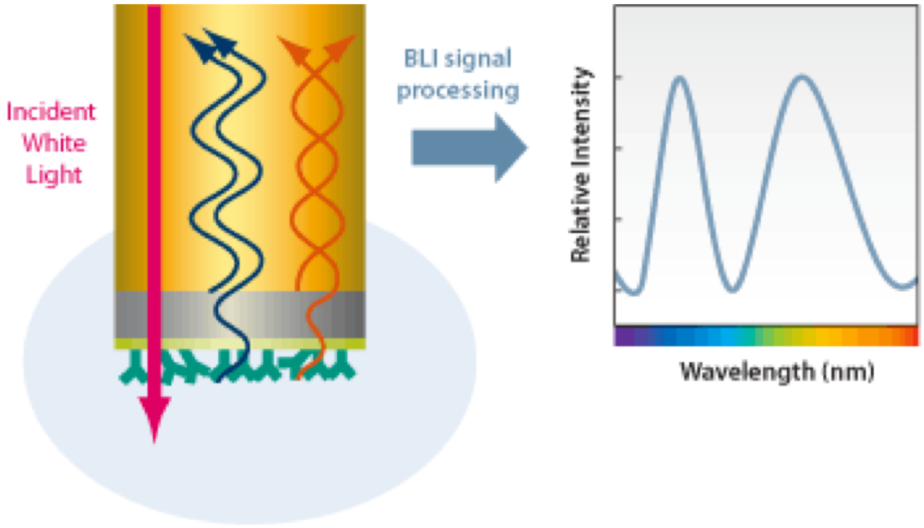
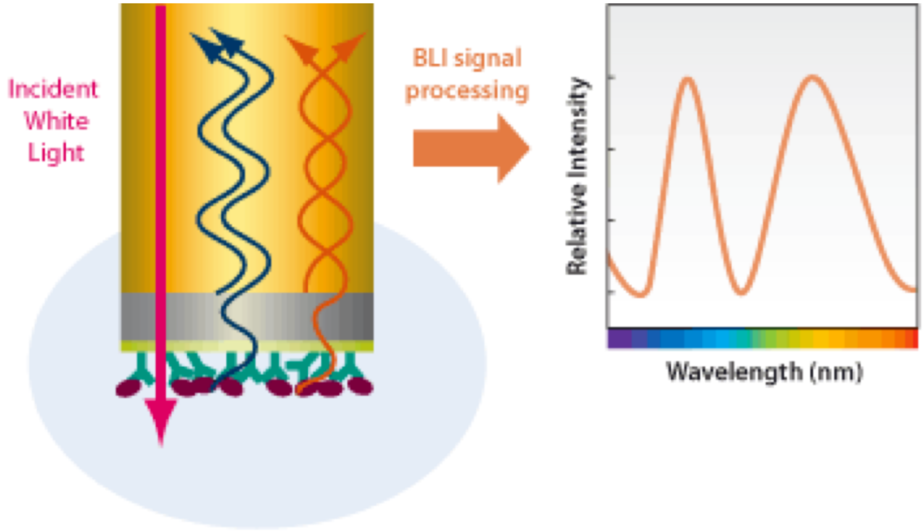
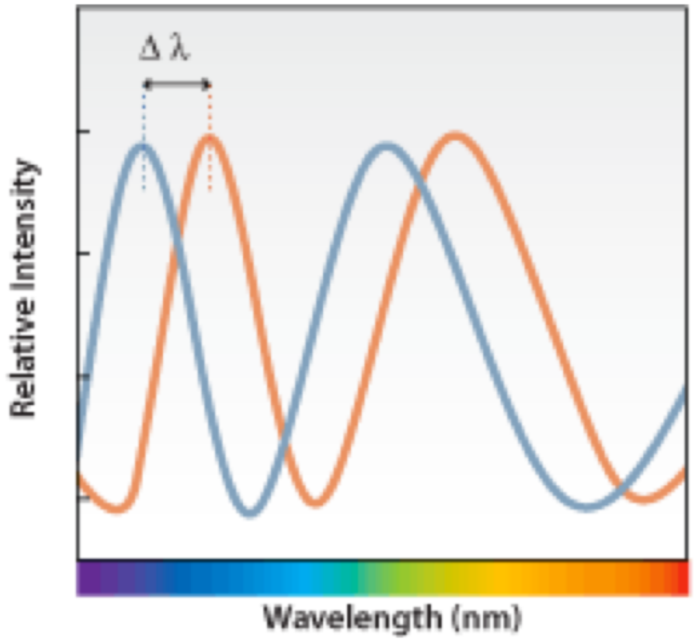


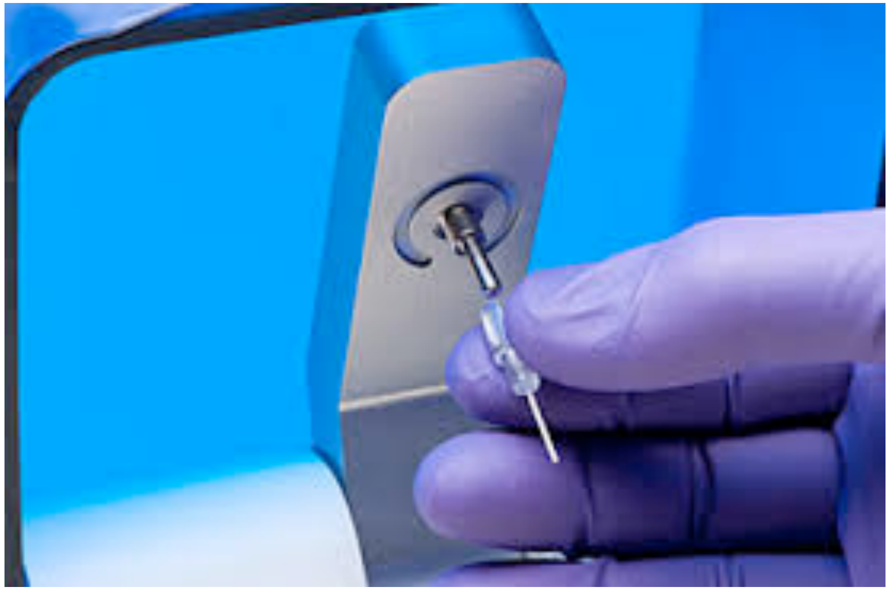
Figure 1

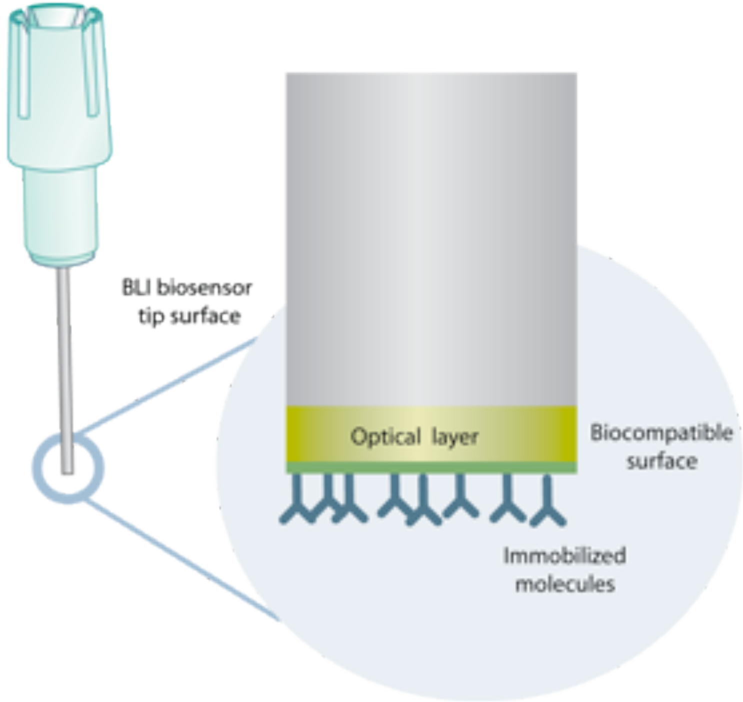




The binding between a ligand immobilized on the biosensor tip surface and an analyte in solution produces an increase in optical thickness at the biosensor tip, which results in a wavelength shift, Δλ, which is a direct measure of the change in thickness of the biological layer.

Dip and Read™ Biosensors

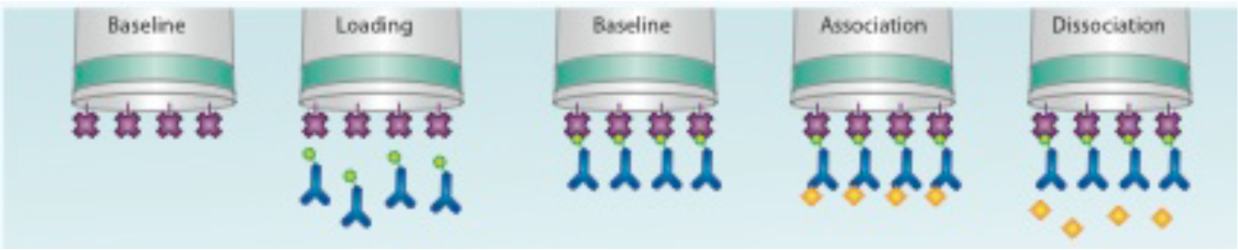


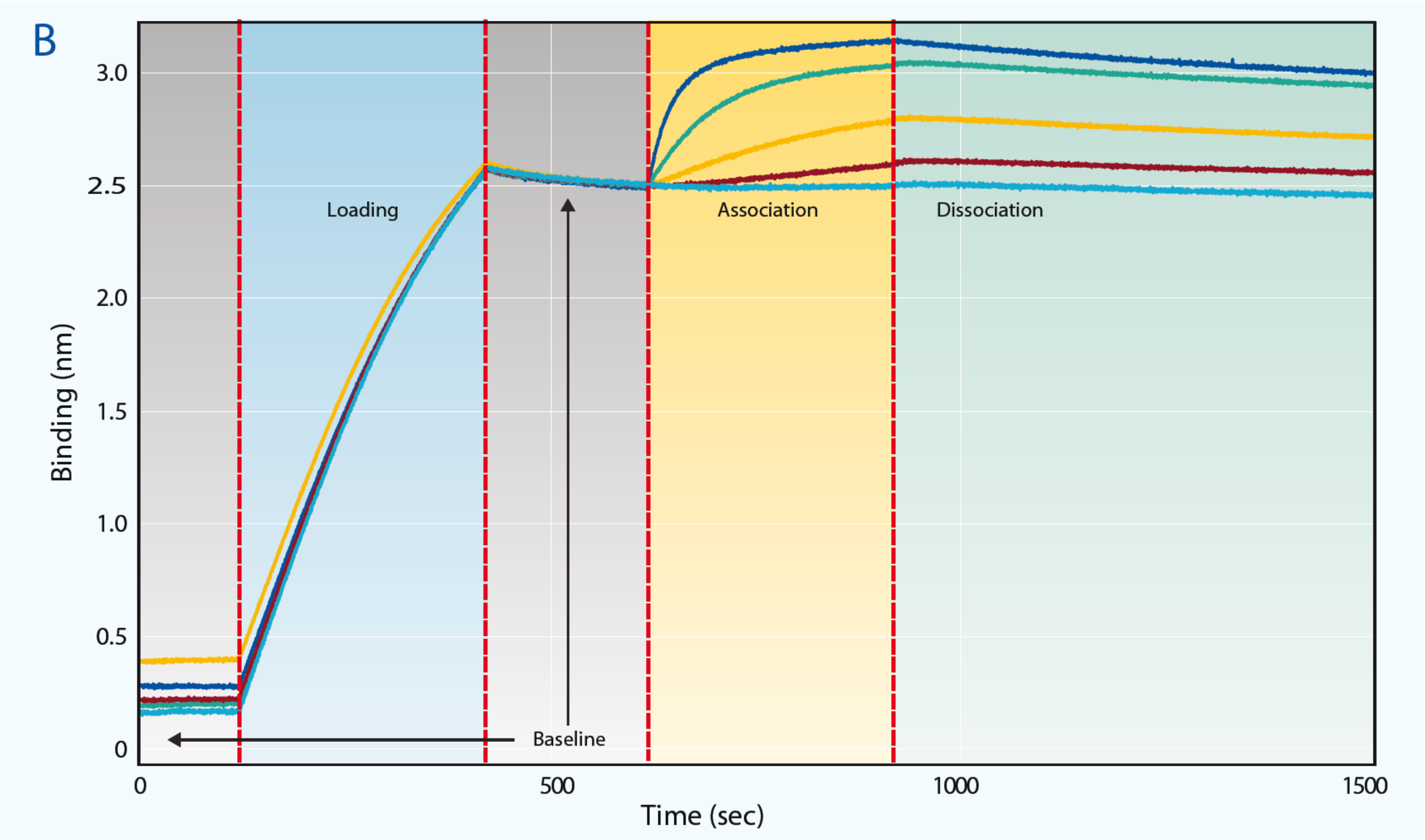


Direct immobilisation: amine coupling, biotin intercation with streptavidin

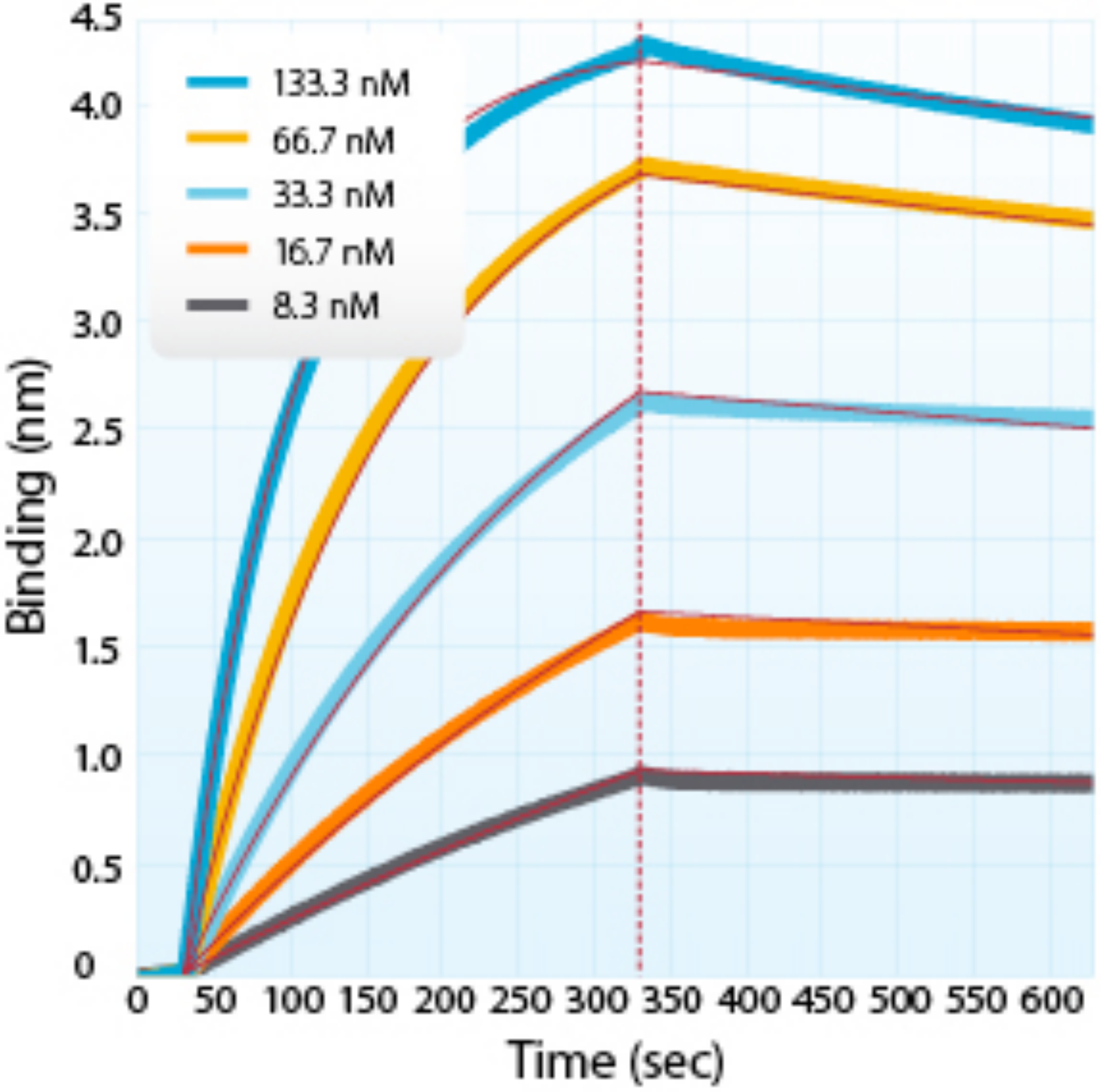
Capture-based approach: high affinity capture antibody (anti-FC, anti-His) or use of known motif or tag (protein A, Ni NTA)

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 excatly	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 120k€ 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 x K_D
Ligand 60 µl concentration = 100 x K_D

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

Thermophorèse Labeled protein 100 µl 20 µM
Ligand 20 µl concentration = 40-50 x K_D

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

Review Article

Journal of Molecular Recognition

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(www.interscience.wiley.com) DOI:10.1002/jmr.928

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

afmb
architecture et fonction
des macromolécules biologiques

Emmanuel Nonny

STALLERGENES

Hans de Haard

arGEN-X

Biacore T200

Octet Red 96

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€

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

NO MAINTENANCE

Experimental protocols for determination of kinetic constants

Biacore T200
(plateform)

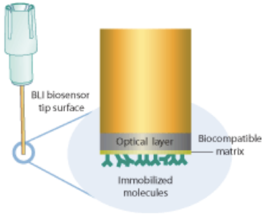
« Standard » protocol
- Ab immobilization
- choice of sensor chip : CM5 type => amine coupling
- pH scouting for Ab immobilization
- immobilization of Ab using target level wizard for theoretical Rmax 50-100 RU
- <u>regeneration conditions</u> (to dissociate Ab-Ag complex)
- repetition of injections 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 µg/ml) about 1 to 1.5 nm

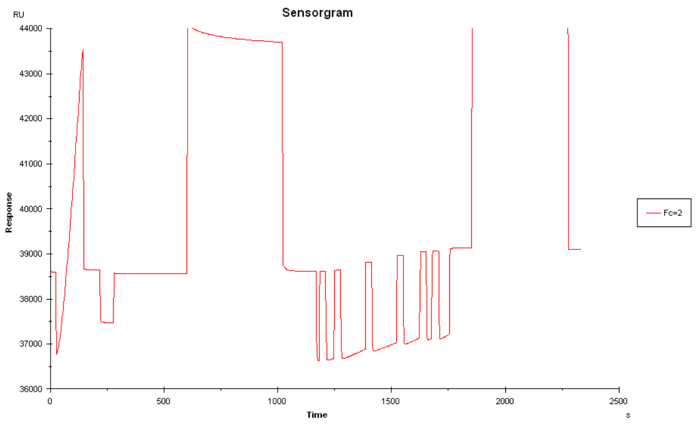
- Ag as analyte in 200 µl wells (can be recovered)



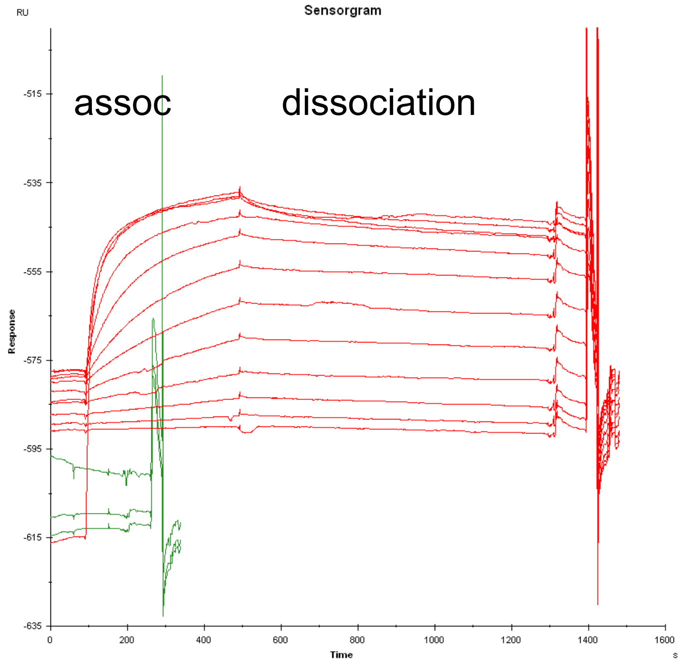
Biacore T200

OctetRed

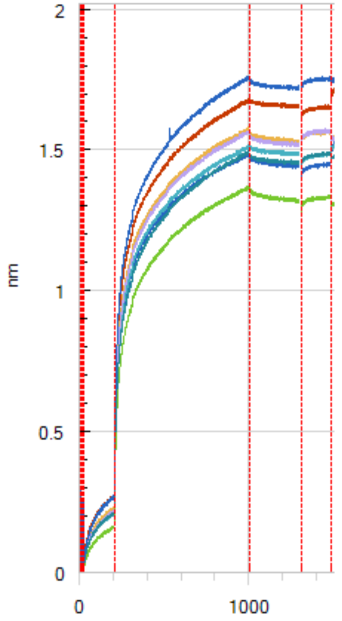
Immobilization



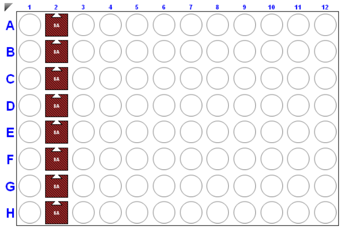
all sensorgrams



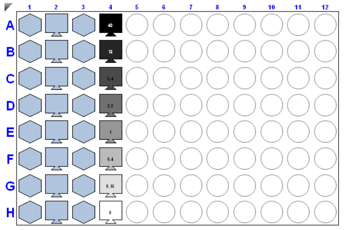
loading onto AHC biosensors



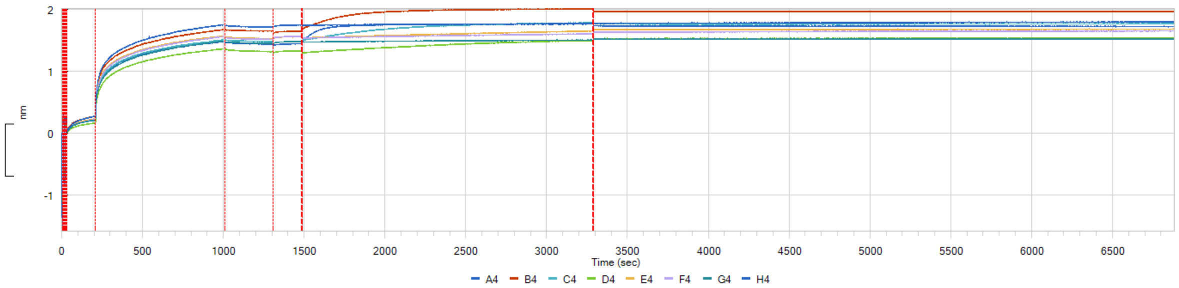
biosensors



wells

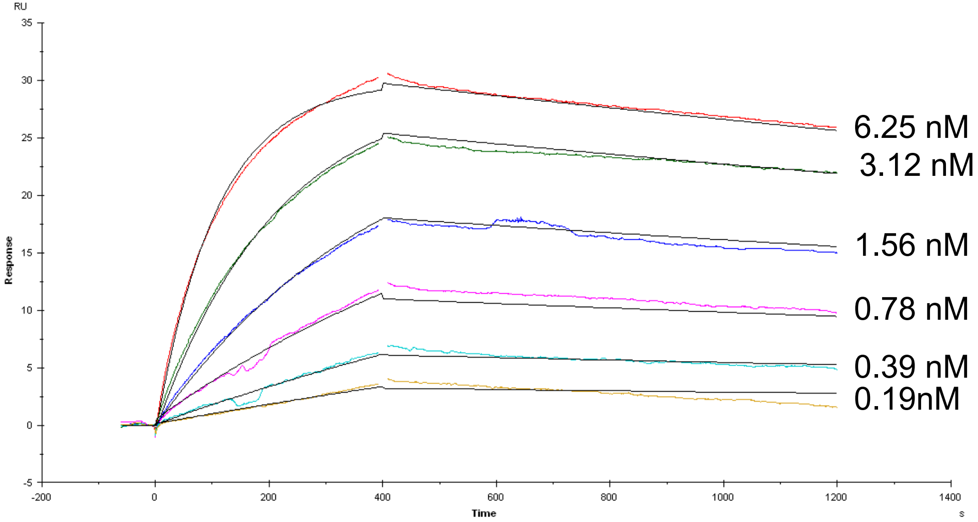


load association dissociation



Kinetics constants for Example 1 (same K_D)

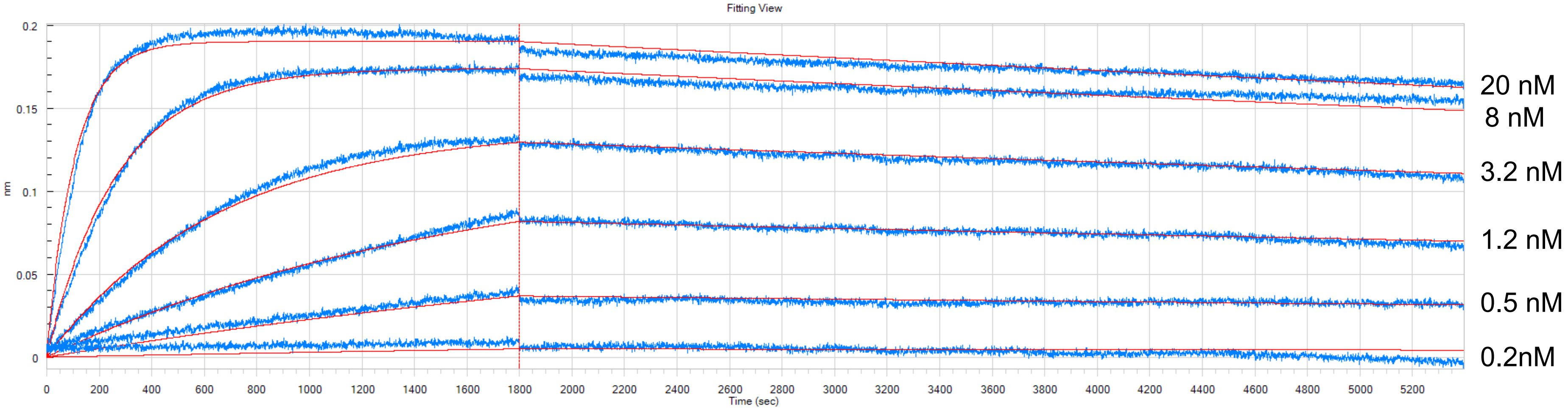
conc of Ag : 0.048 to 6.25 nM



K_D (nM)	Kass ($M^{-1}s^{-1}$)	kdiss (s^{-1})
0.13	1.4×10^6	1.9×10^{-4}

K_D (nM)	Kass ($M^{-1}s^{-1}$)	kdiss (s^{-1})
0.1	4.5×10^5	4.6×10^{-5}

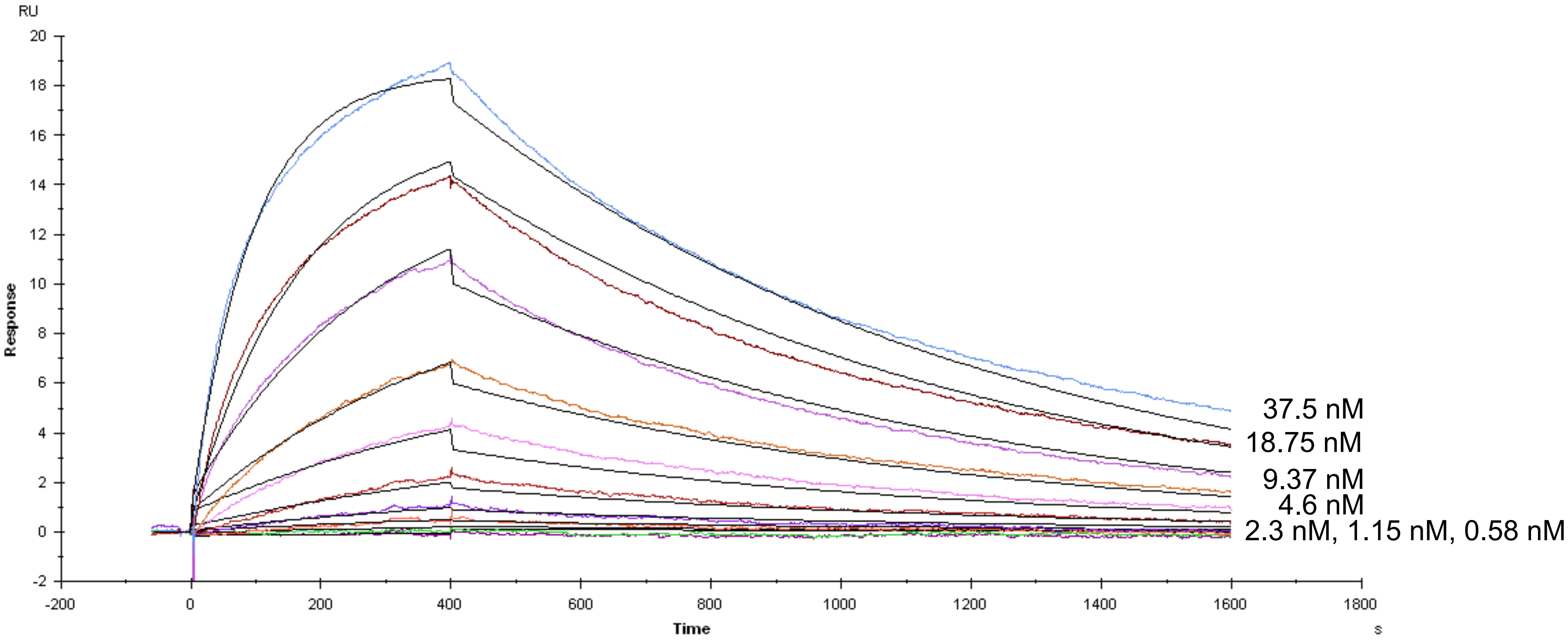
Load Ab onto AHC biosensors, conc of Ag : 0.2 to 20 nM



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

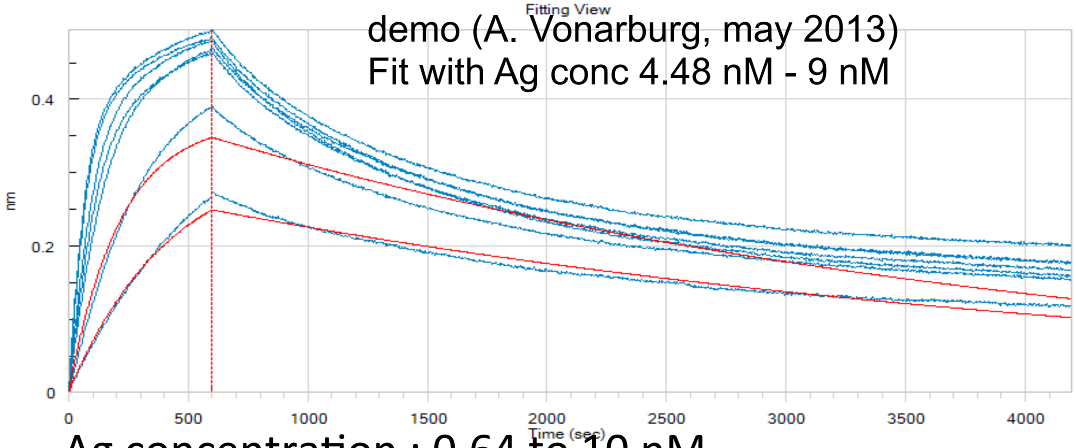
Ag concentration : 0.58 -37.5nM

(biacore T200)



K_D (nM)	Kass ($M^{-1}s^{-1}$)	kdiss (s^{-1})
5.9	2 x 10^5	1.2 x 10^{-3}

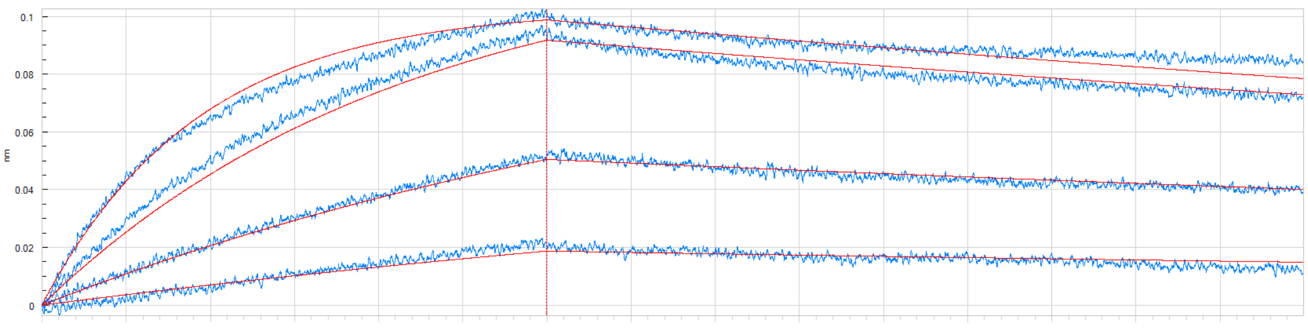
Kinetics constant for Example 2
(three different experiments with OctetRed)



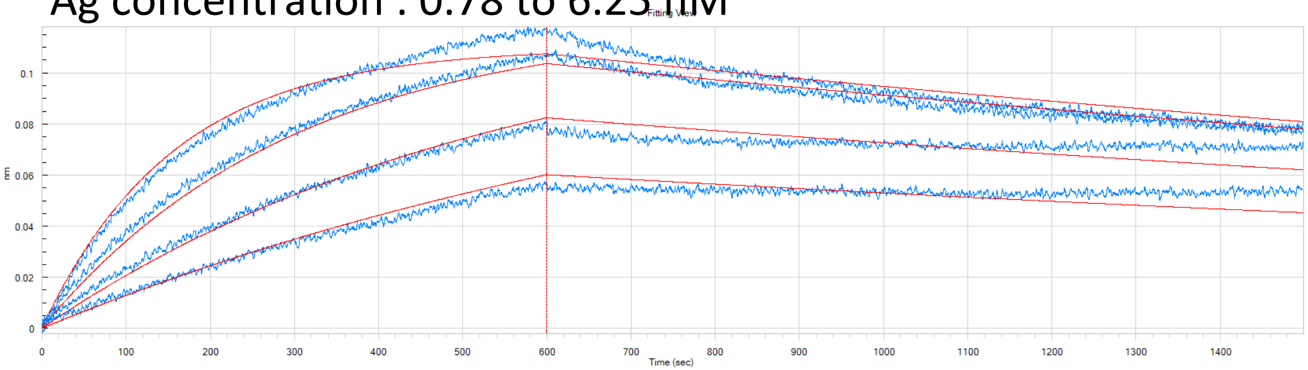
demo (A. Vonarburg, may 2013)
Fit with Ag conc 4.48 nM - 9 nM

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

Ag concentration : 0.64 to 10 nM



Ag concentration : 0.78 to 6.25 nM



K_D (nM)
0.57
0.46
0.37

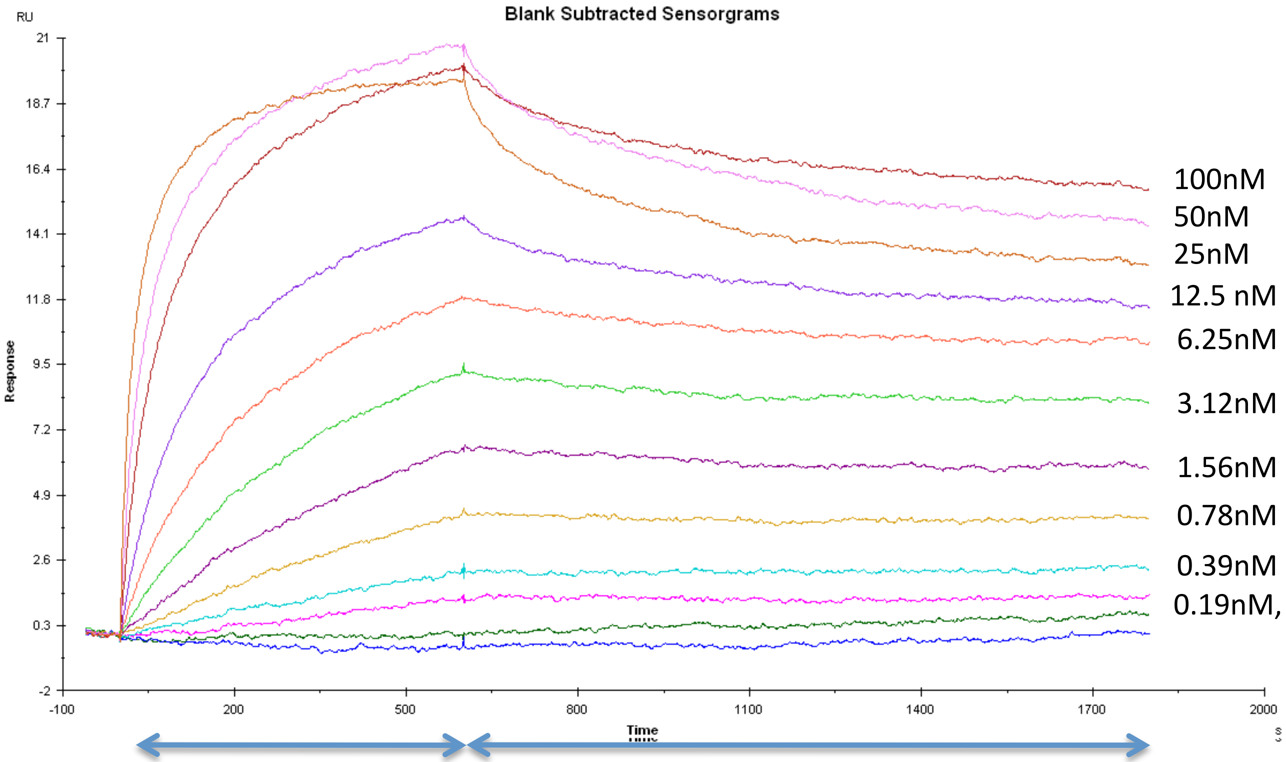
Comparison of K_D constants from BiacoreT200 and OctetRed96

	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)



100nM
50nM
25nM
12.5 nM
6.25nM
3.12nM
1.56nM
0.78nM
0.39nM
0.19nM, 0.096nM, 0.048nM

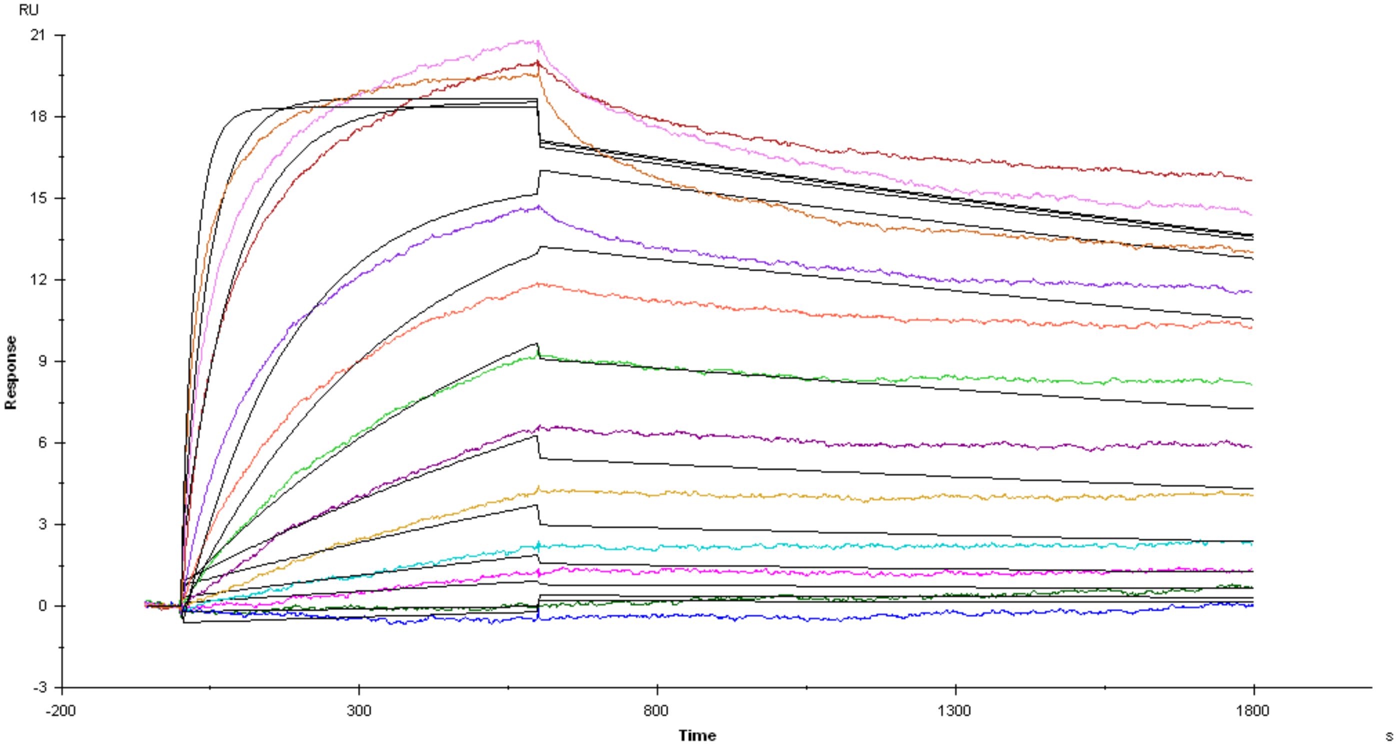
Rmax

Req

assoc 600 s

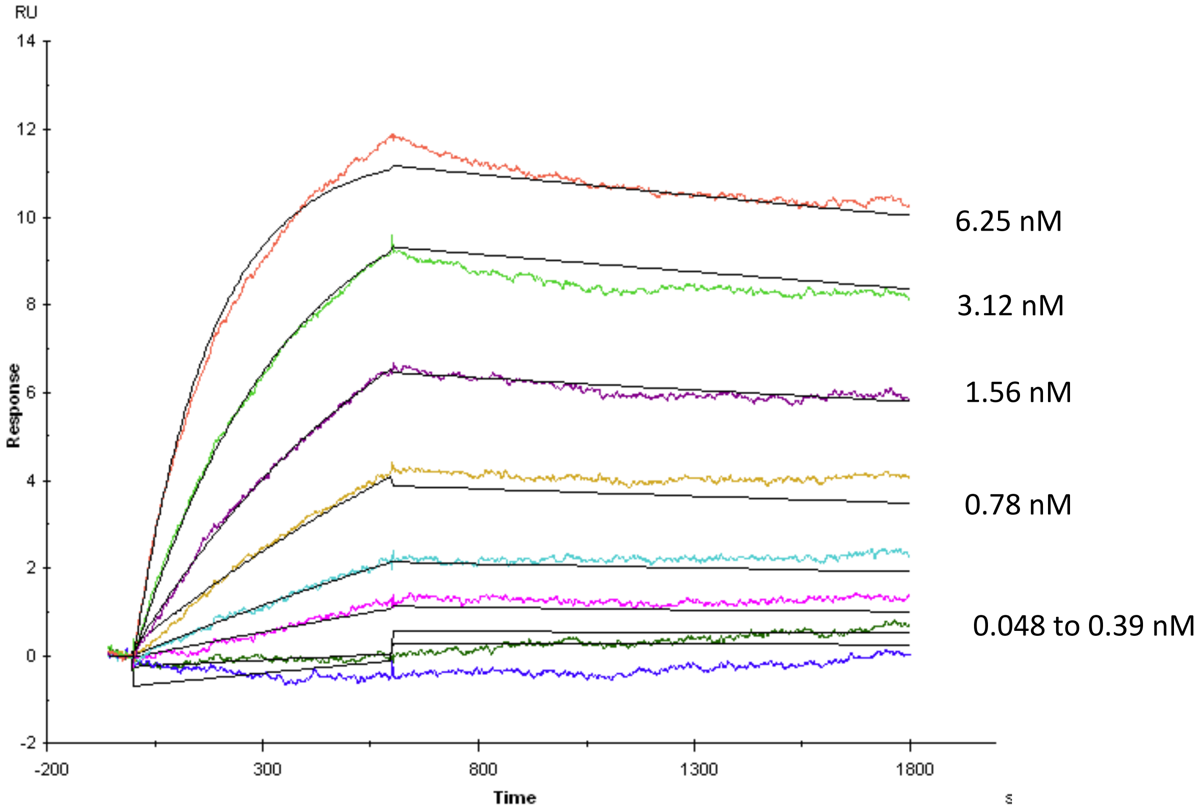
dissoc 1200 s

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



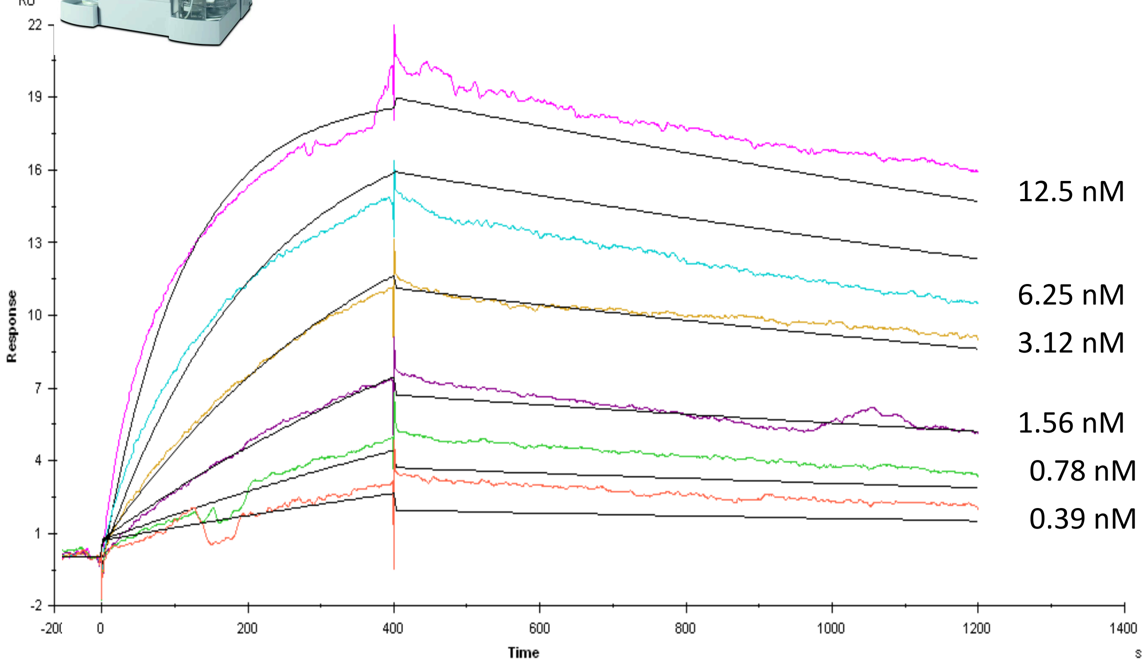
Bad fitting, KD = 0.43 nM

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

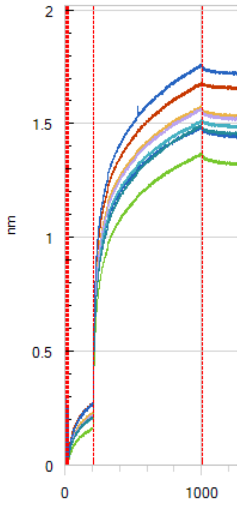


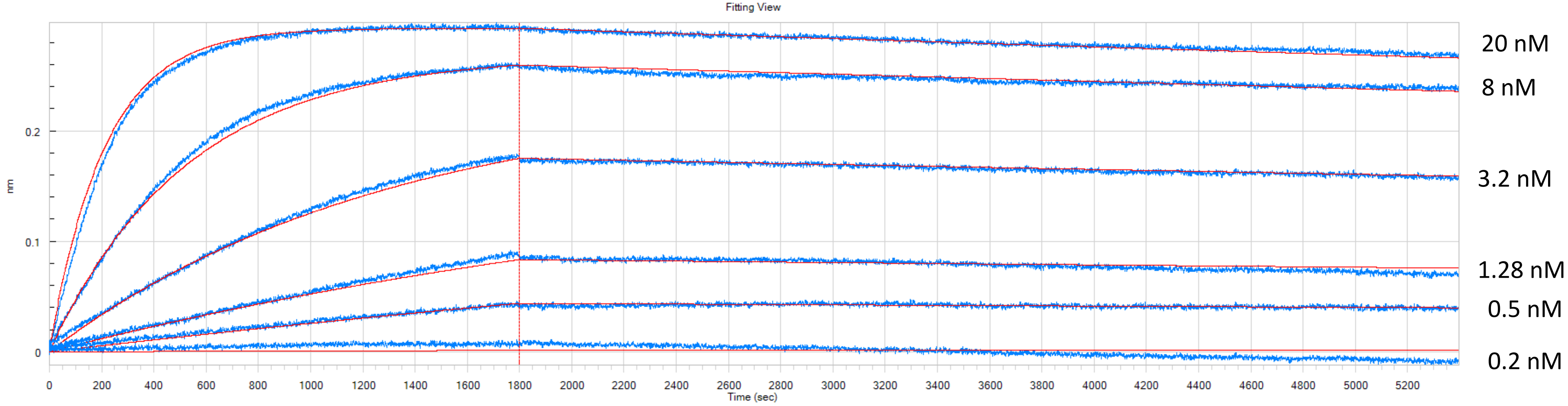
K_D (nM)	Kass($M^{-1}s^{-1}$)	Kdiss(s^{-1})
0.1	8.8×10^5	9×10^{-5}

Example 4 : different dissociation constants using biacore and OctetRed

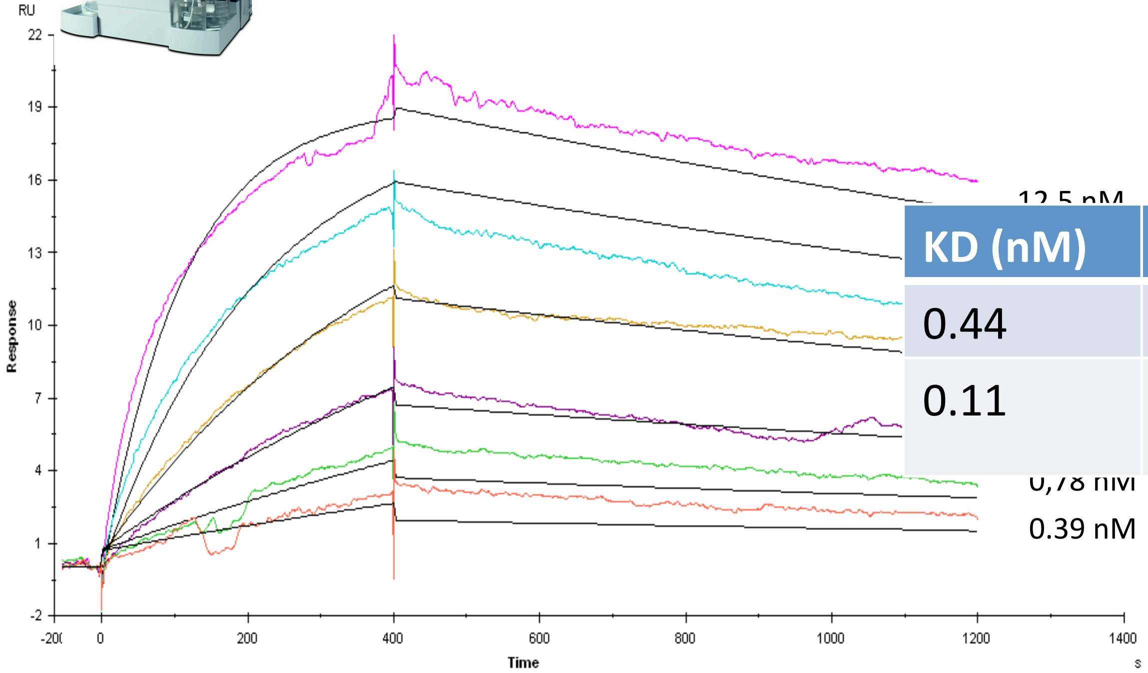


Ab load onto AHC biosensors



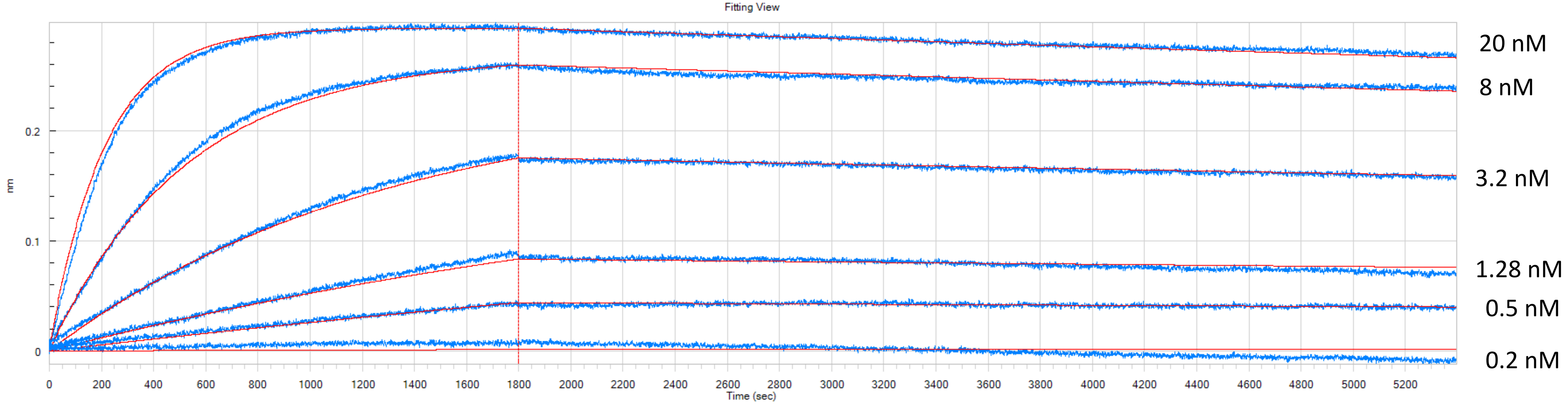


Example 4 : different dissociation constants using biacore and OctetRed

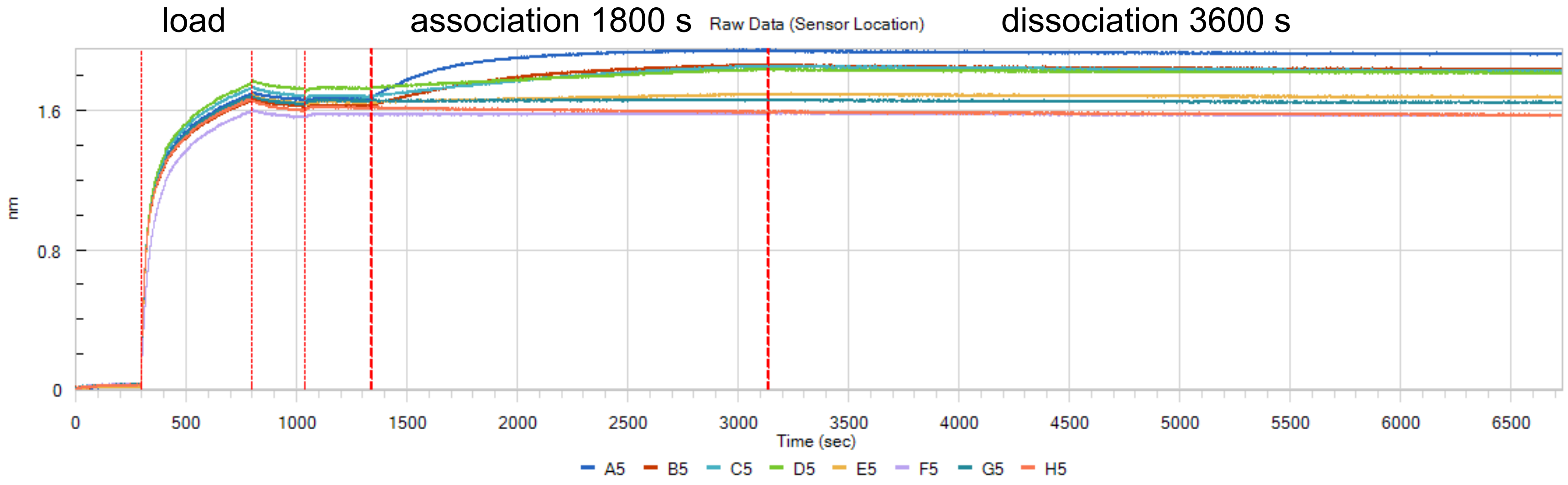


KD (nM)	Kass ($M^{-1}s^{-1}$)	Kdiss (s^{-1})	
0.44	7 x 10^5	3 x 10^{-4}	biacore
0.11	2.33 x 10^5	2 x 10^{-5}	octetRed

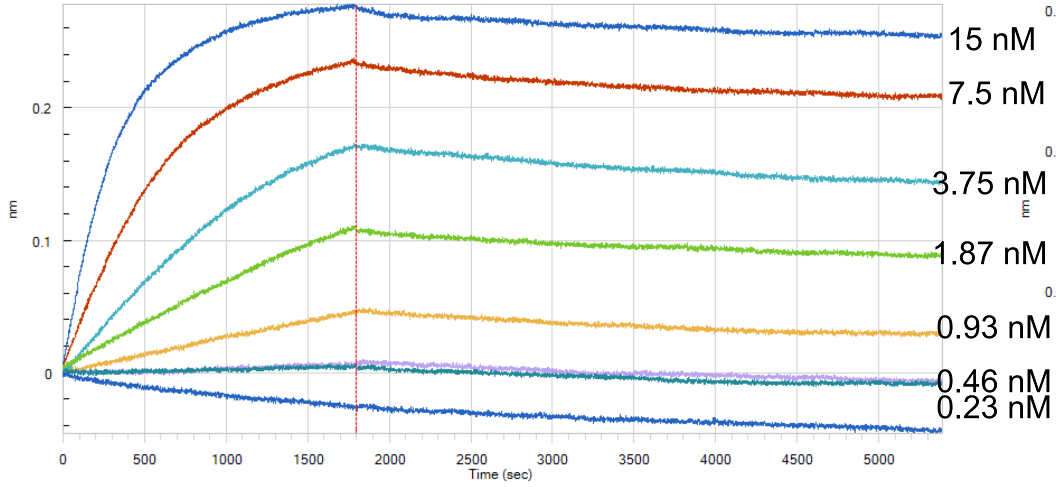
lower with octet (x15)



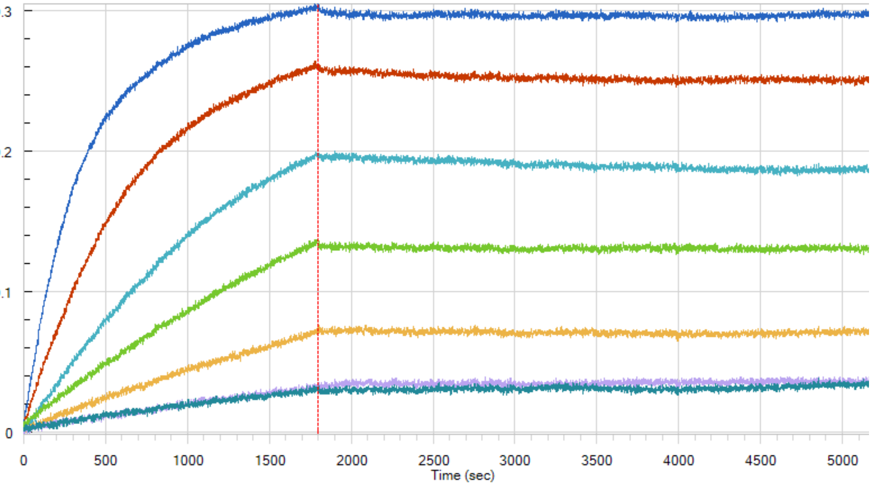
Example 5 : tight complex (K_D around 10^{-11} M)



Ag concentration 0.23 nM to 15 nM Ag

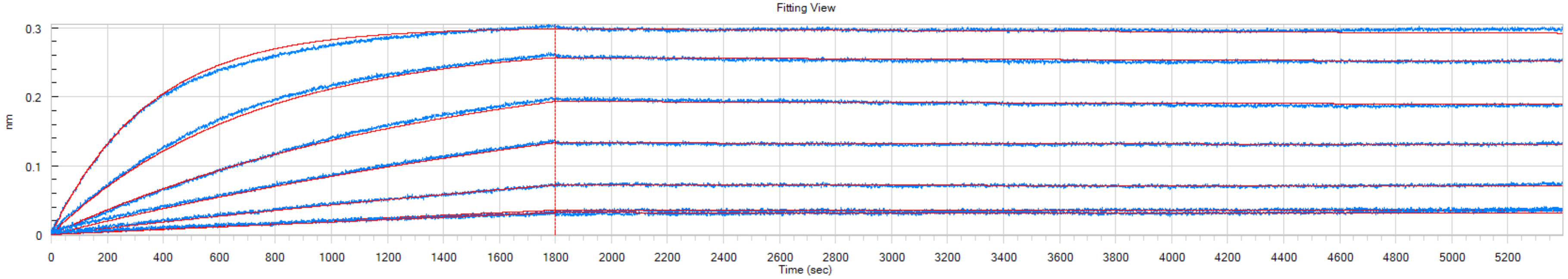


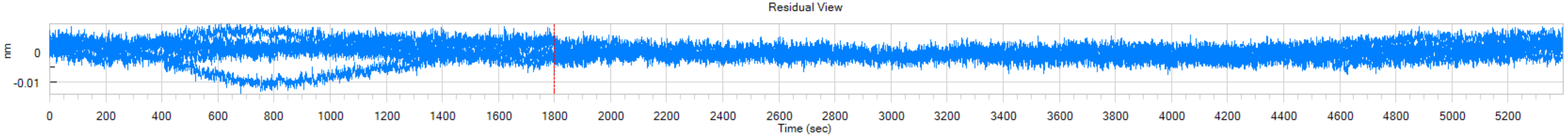
Blank (reference well) substracted

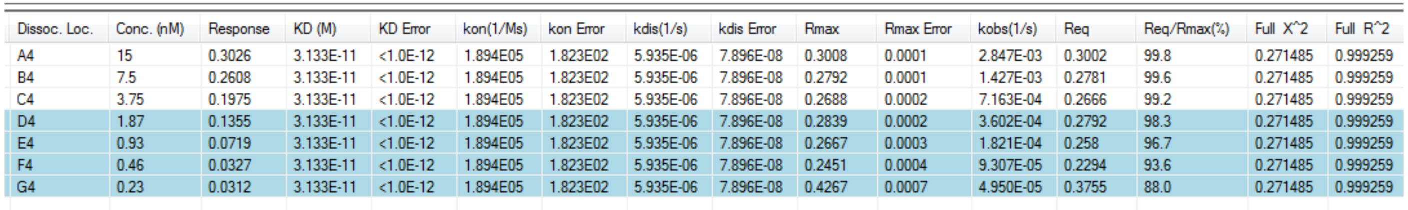


Example 5, fitting of the data model 1:1

Ag concentration 0.23 nM to 15 nM Ag





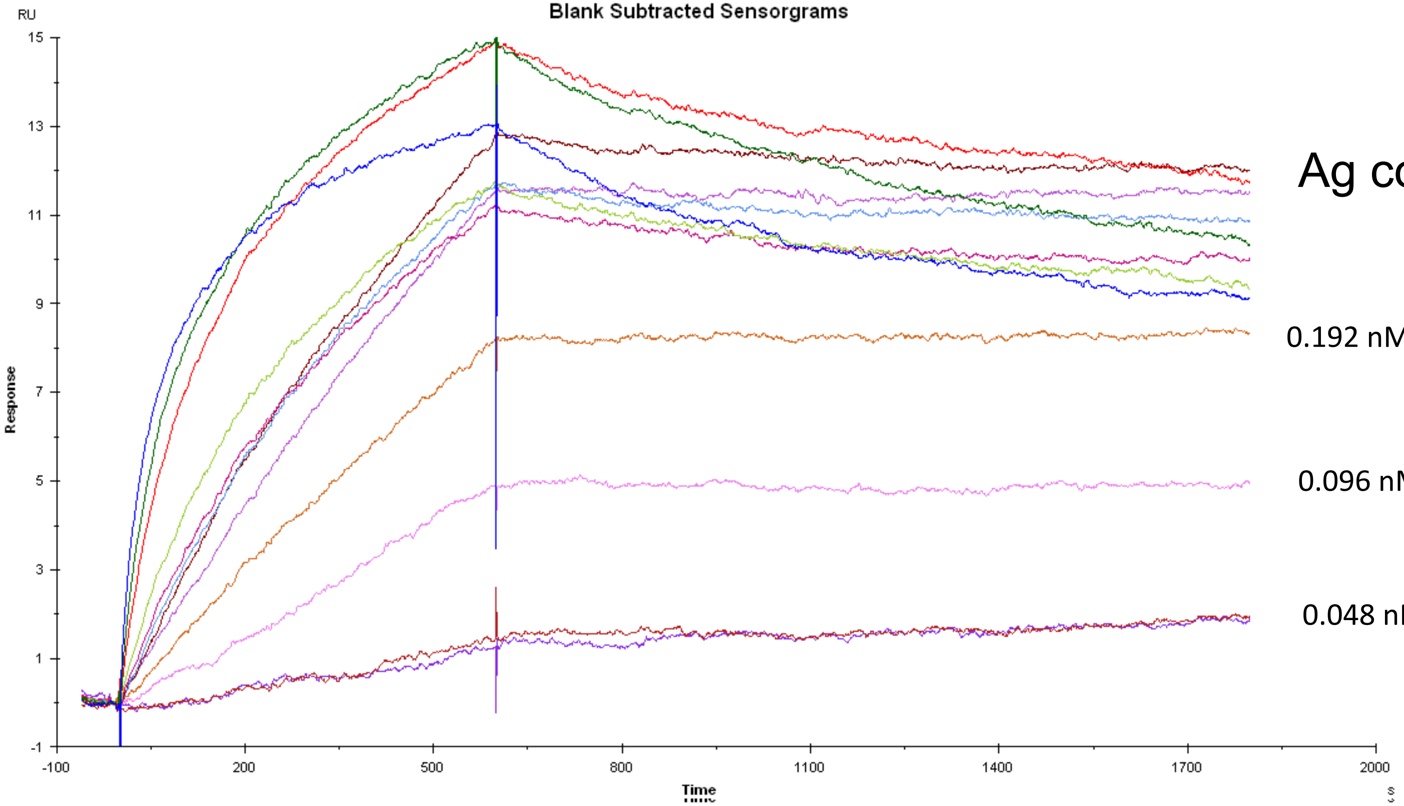


Dissoc. Loc.	Conc. (nM)	Response	KD (M)	KD Error	kon(1/Ms)	kon Error	kdis(1/s)	kdis Error	Rmax	Rmax Error	kobs(1/s)	Req	Req/Rmax(%)	Full X^2	Full R^2
A4	15	0.3026	3.133E-11	<1.0E-12	1.894E05	1.823E02	5.935E-06	7.896E-08	0.3008	0.0001	2.847E-03	0.3002	99.8	0.271485	0.999259
B4	7.5	0.2608	3.133E-11	<1.0E-12	1.894E05	1.823E02	5.935E-06	7.896E-08	0.2792	0.0001	1.427E-03	0.2781	99.6	0.271485	0.999259
C4	3.75	0.1975	3.133E-11	<1.0E-12	1.894E05	1.823E02	5.935E-06	7.896E-08	0.2688	0.0002	7.163E-04	0.2666	99.2	0.271485	0.999259
D4	1.87	0.1355	3.133E-11	<1.0E-12	1.894E05	1.823E02	5.935E-06	7.896E-08	0.2839	0.0002	3.602E-04	0.2792	98.3	0.271485	0.999259
E4	0.93	0.0719	3.133E-11	<1.0E-12	1.894E05	1.823E02	5.935E-06	7.896E-08	0.2667	0.0003	1.821E-04	0.258	96.7	0.271485	0.999259
F4	0.46	0.0327	3.133E-11	<1.0E-12	1.894E05	1.823E02	5.935E-06	7.896E-08	0.2451	0.0004	9.307E-05	0.2294	93.6	0.271485	0.999259
G4	0.23	0.0312	3.133E-11	<1.0E-12	1.894E05	1.823E02	5.935E-06	7.896E-08	0.4267	0.0007	4.950E-05	0.3755	88.0	0.271485	0.999259

KD (nM)	Kass($M^{-1}s^{-1}$)	Kdiss(s^{-1})
0.031	1.89×10^5	5.9×10^{-6}

Example 6 : very tight complex (K_D around 10^{-12} M)

Biacore T200



Ag concentration

0.192 nM

0.096 nM

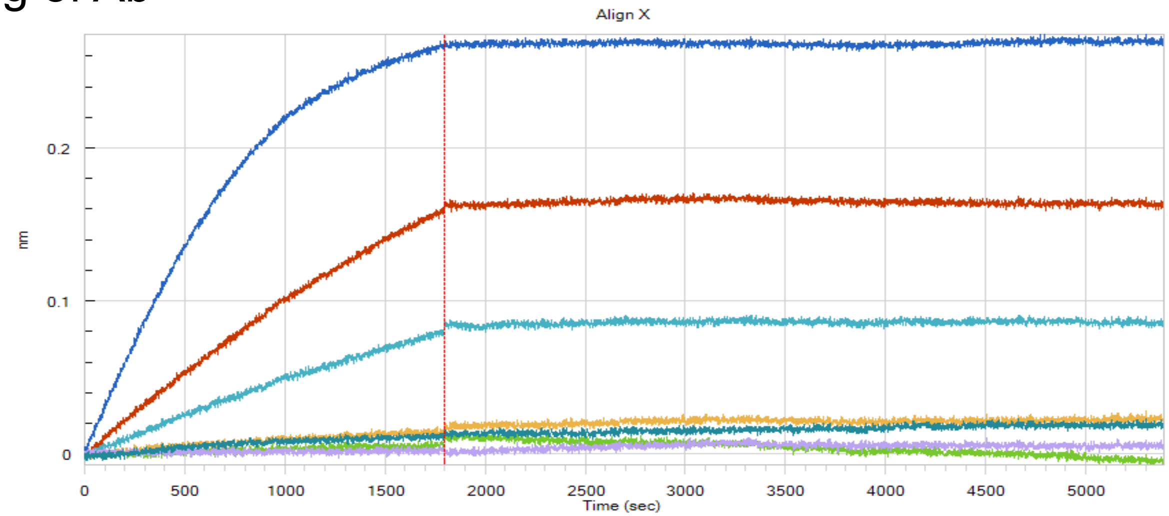
0.048 nM

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

NO DATA FIT POSSIBLE
(needs for protocol optimization)

Example 6 : kinetic experiment on AHC biosensors using octetRed96

loading of Ab



5 nM

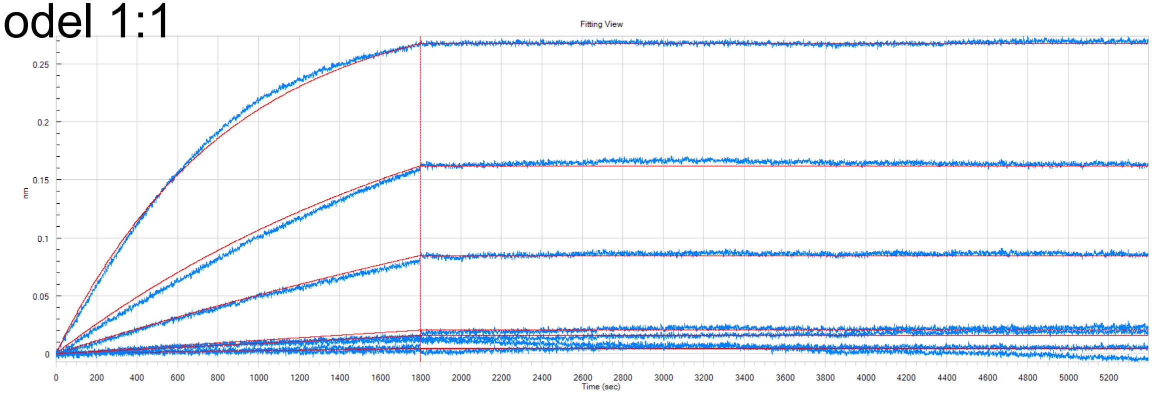
2 nM

0.8 nM

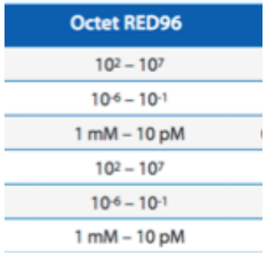
0.32 nM, 0.128 nM
0.051 nM, 0.02 nM

assoc 1800s
dissoc 3600s

Fit model 1:1

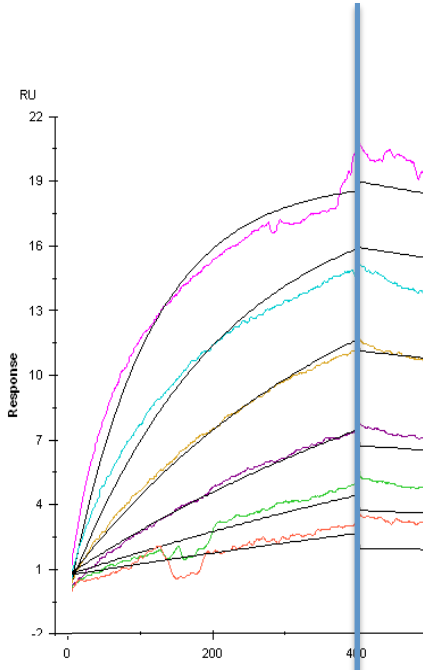


increase
dissociation
time ?



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

KD (nM)	Kass($M^{-1}s^{-1}$)	Kdiss(s^{-1})
< 0.001	2.35×10^5	$< 10^{-7}$



SPR : non optimized protocols (standard protocols)

association time (Kass)

SPR : too short association time (400s) to reach Req

flow rate x contact time/60 = 2-350

for 1800 s association « low » flow rate, 12µl/min

Problems with long asso/disso times with SPR:

- to avoid mass transport, flow rate must be >= 30µl/min

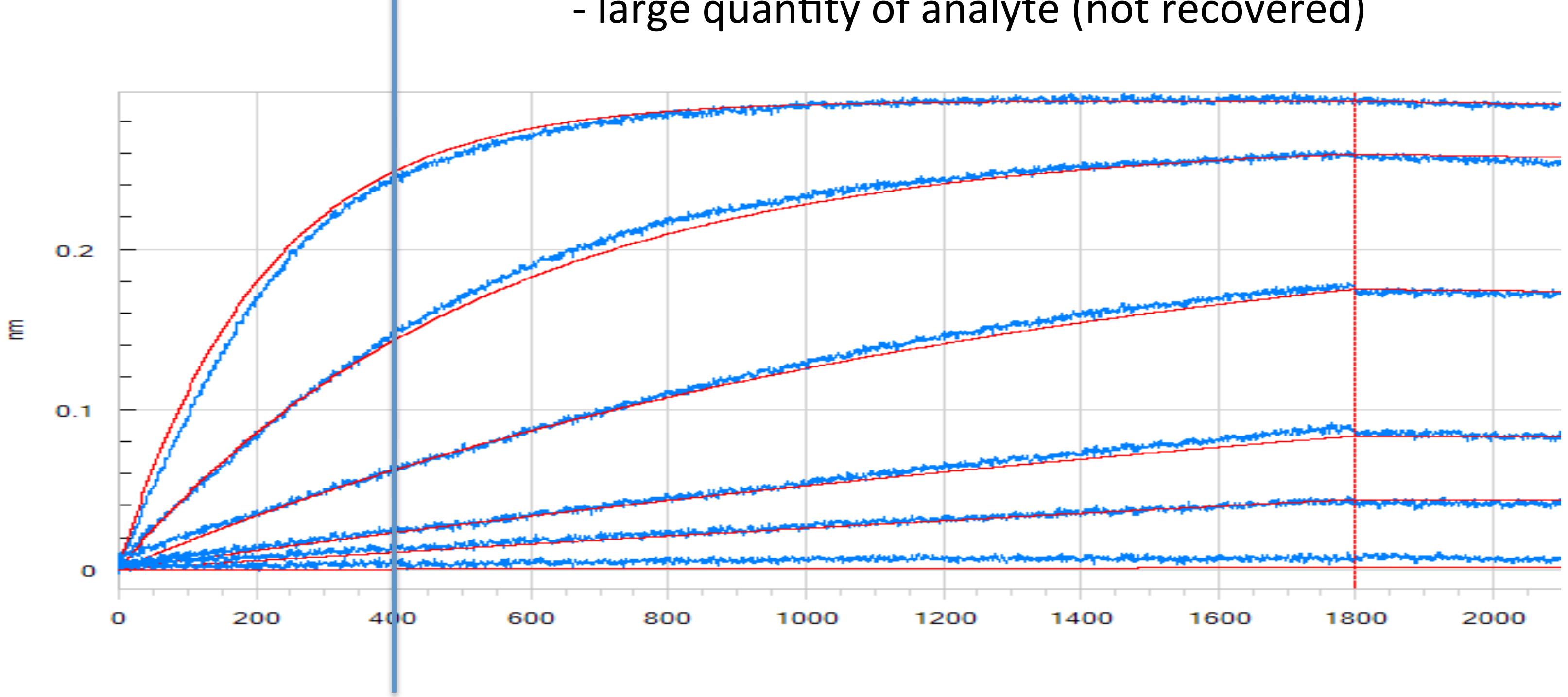
  maximum association time= 700 s

- experience duration for 1800 s (assoc) and 8000 s (dissoc)

  BLI : about 3 hours for 8 concentrations

  SPR : about 32 hours for 8 concentrations

- large quantity of analyte (not recovered)



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