Surface Plasmon Resonance BioLayer Interferometry

"label-free" and real-time affinity & kinetics measurements

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Institut de Biologie Structurale

Complement and Antibodies In Diseases

Integrated Structural Biology

SPR/BLI platform

France

SPR/BLI: introduction

Objectives

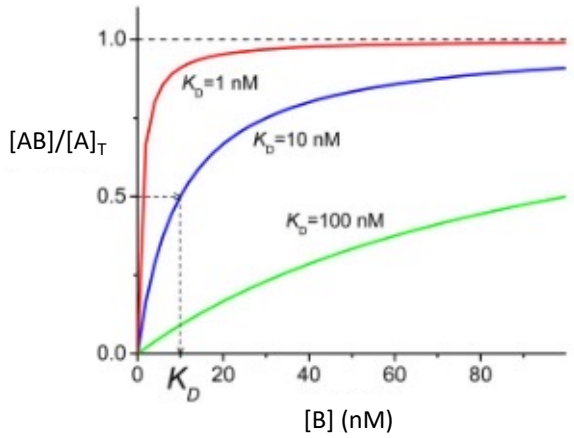
- The SPR & BLI technologies for biomolecular interactions
- A few examples in Glycosciences
- A demonstration

Introduction

Affinity and kinetics?

- The affinity constant:
  - K_A = equilibrium Association constants (M^{-1})
  - K_D = equilibrium Dissociation constants (M)

$$A + B \underset{k_d}{\overset{k_a}{\rightleftharpoons}} AB$$



$$\text{association} = \text{dissociation}$$

$$k_a.[A].[B] = k_d.[AB]$$

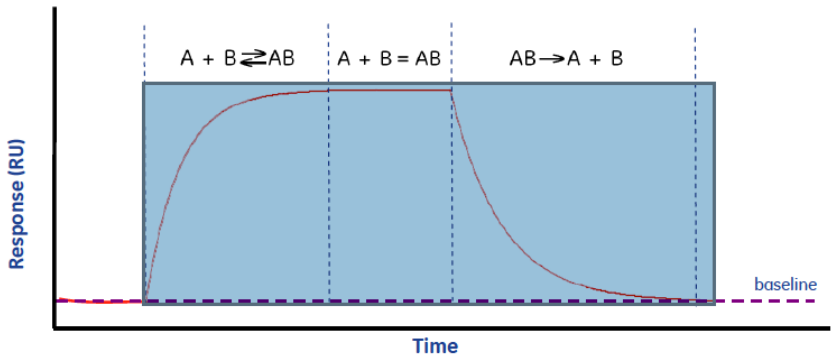
$$K_D = \frac{1}{K_A} = \frac{[A].[B]}{[AB]}$$

Introduction

Affinity and kinetics?

- The rate of kinetics:
  - k_a or k_{on} = association rate constant ($M^{-1}.s^{-1}$)
  - k_d or k_{off} = dissociation rate constant (s^{-1})

$$A + B \underset{k_d}{\overset{k_a}{\rightleftharpoons}} AB$$



$$\text{association:} \quad \frac{d[AB]}{dt} = k_a.[A].[B]$$

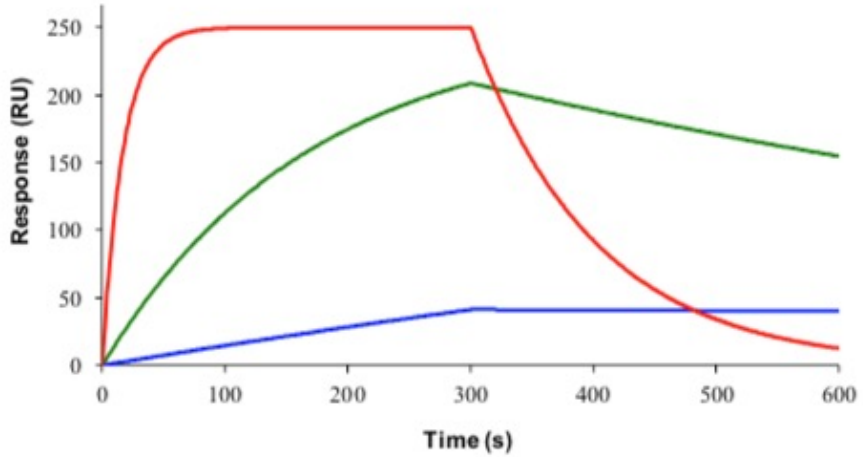
$$\text{dissociation:} \quad -\frac{d[AB]}{dt} = k_d.[AB]$$

$$\text{net rate:} \quad \frac{d[AB]}{dt} = k_a.[A].[B] - k_d.[AB]$$

Introduction

Affinity and kinetics?

- Characterization of interactions
  - Molecular recognition - Association rate constant (k_a or k_{on}): 10^3 – 10^7 $M^{-1}s^{-1}$
  - Complex stability - Dissociation rate constant (k_d or k_{off}): 5.10^{-6} – 10^{-1} s^{-1}
  - Apparent affinity constant (K_D): 50pM – 100µM
  - The same affinity can be resolved into different kinetic rates



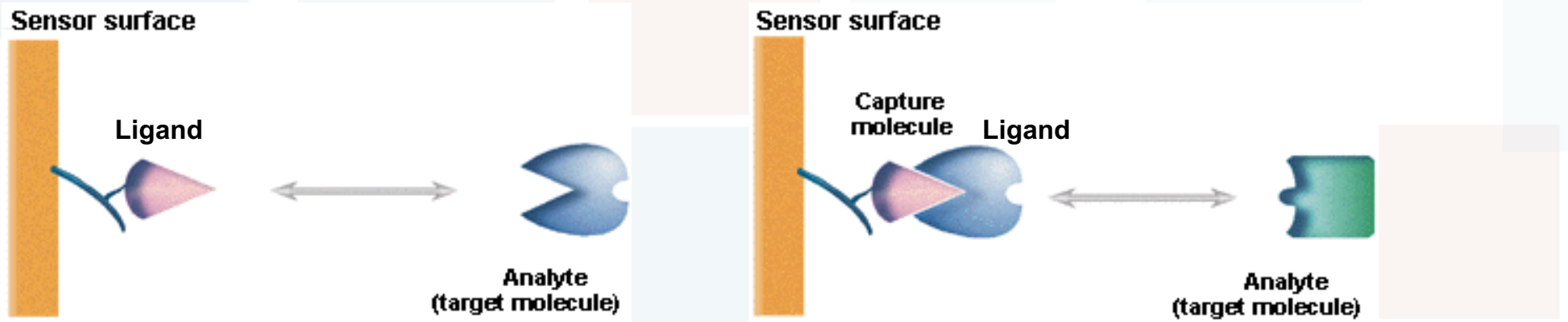
k_a ($M^{-1}.s^{-1}$)	K_d (s^{-1})
10^5	10^{-3}
10^4	10^{-4}
10^6	10^{-2}

$K_D = k_d/k_a = 10^{-8}$ M

Introduction

On-surface methods

- A binding molecule (**ligand**) is immobilized/captured on the sensor
- The target molecule (***analyte***) is put in contact binds to the ligand



Introduction

Experimental design

Sample preparation & QC

↓

Surface preparation

↓

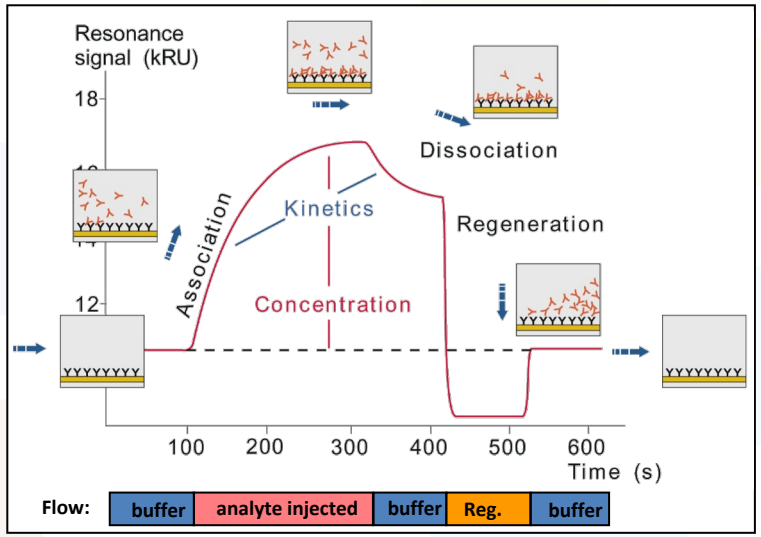
Sample contact

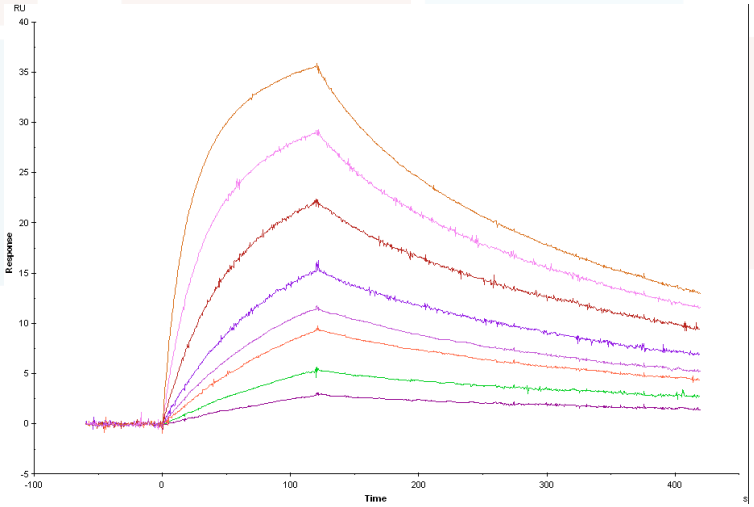
↓

Regeneration

↓

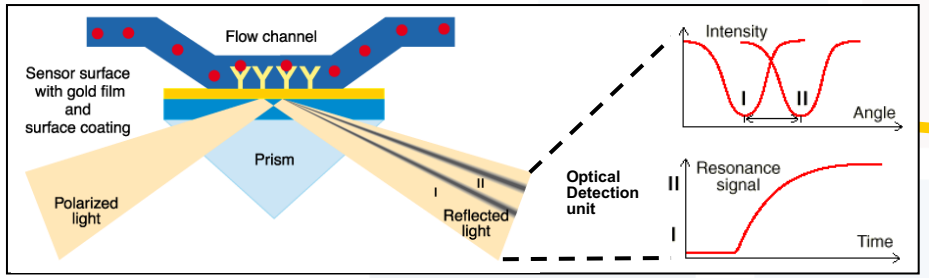
Analysis & Evaluation



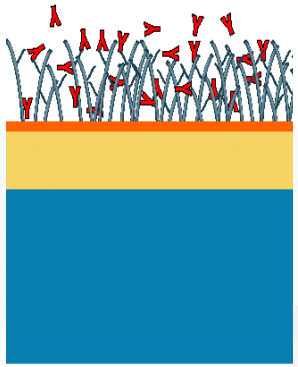


SPR

The Biacore technology



SPR Detection System



immobilized biomolecule

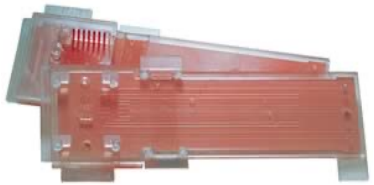
dextran layer (~ 100 nm)

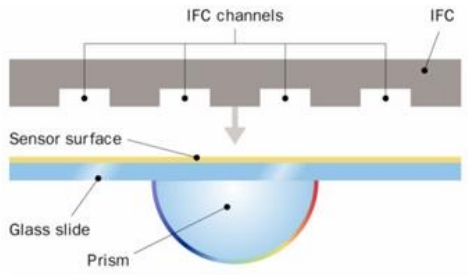
linker layer

goldlayer (~ 50 nm)

glass

Sensorchip



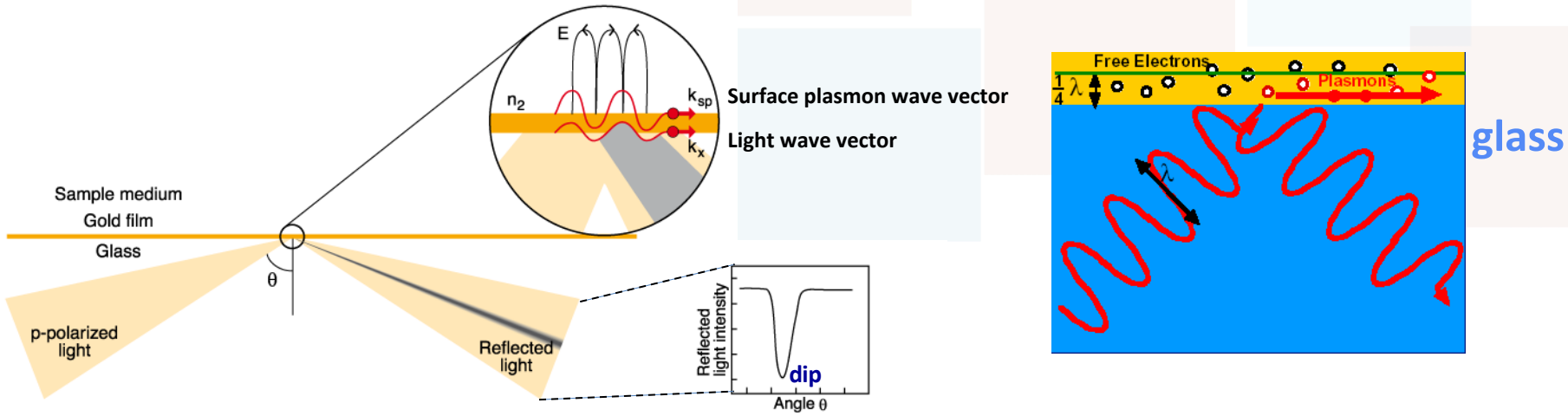


Microfluidic System

SPR

Basis of SPR measurements

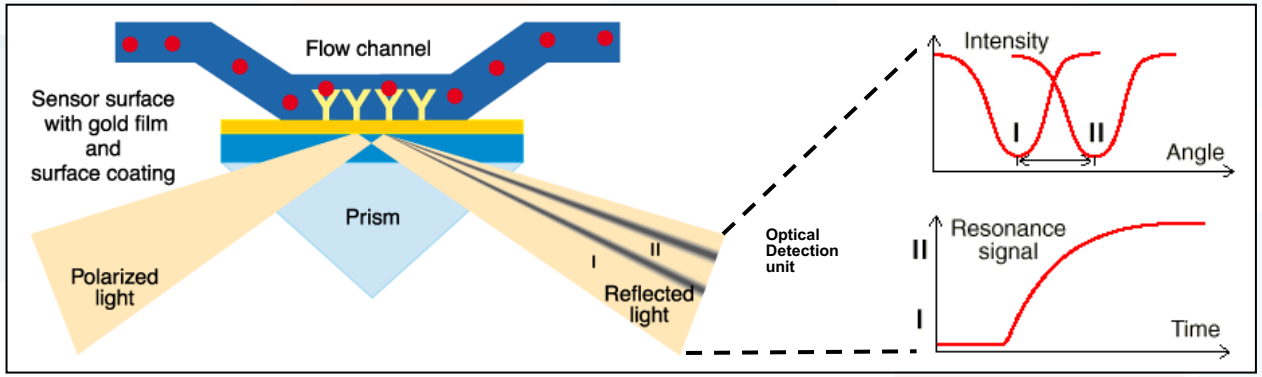
- A EM component, the evanescent waves, enters the low refractive index medium over a short distance from the interface (about one wavelength)
- The interposition of a gold layer generates a resonance phenomenon between free electrons and incident photons, leading to a loss of energy in the reflected light => SPR is detected as a dip in the intensity at a specific angle, which depends of the medium refractive index



SPR

The Biacore technology

- Basis of SPR measurements:

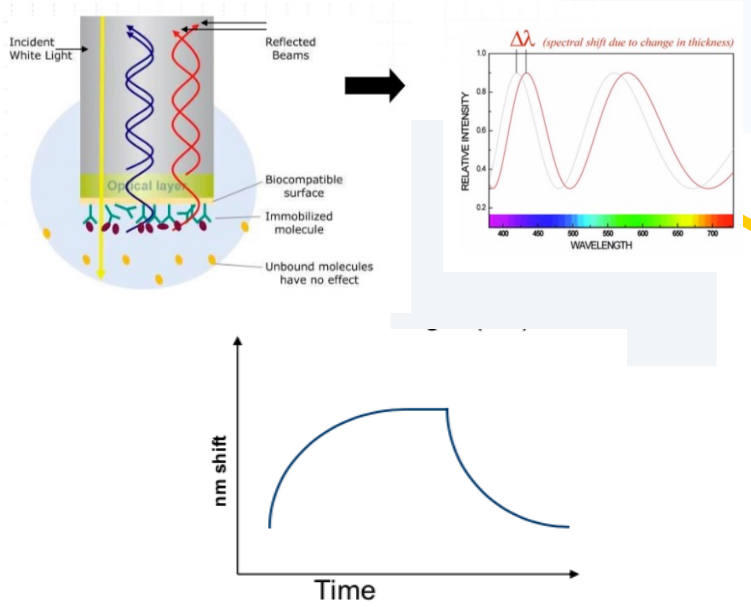


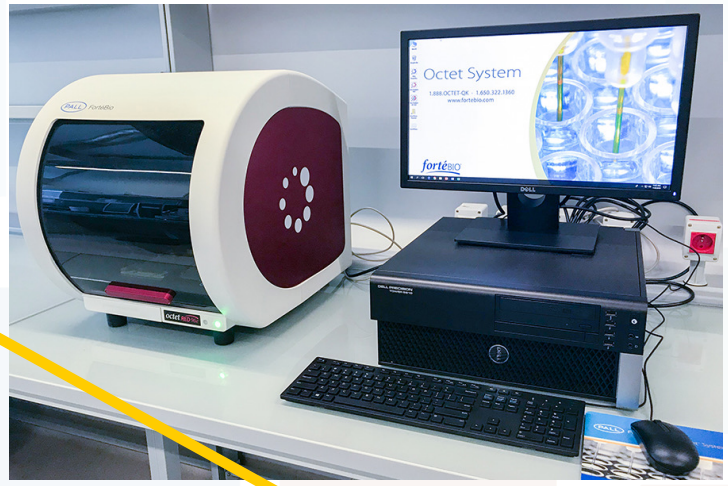
- The optical detector continuously records the position of reduced light intensity and calculates the SPR angle
- Biomolecular binding at the sensor surface changes the refractive index (RI)
  - The SPR angle is changed
  - The SPR angle changes are measured as "response signal units" in real time (RU)

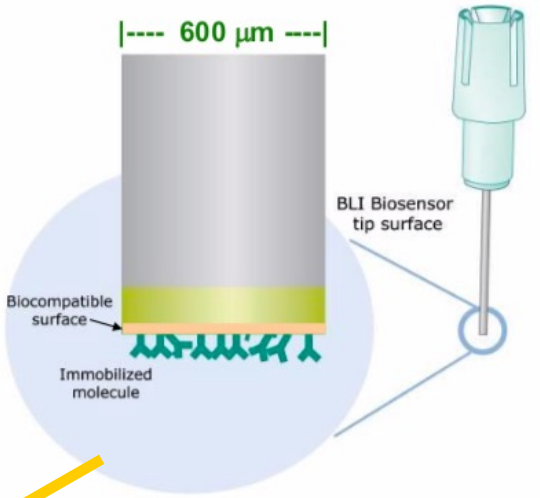
$$1\ RU \Leftrightarrow 10^{-4}\ degre \Leftrightarrow 10^{-6}\ RI \Leftrightarrow 1\ pg\ bound\ mass/mm^2$$

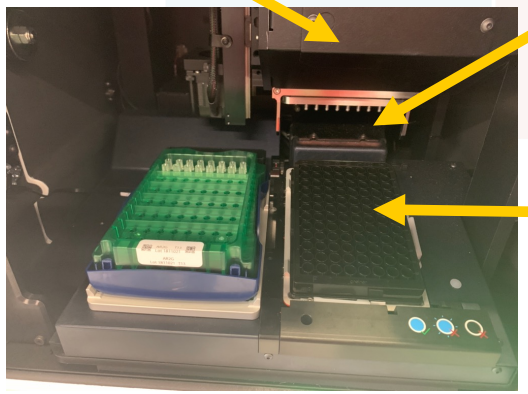
BLI

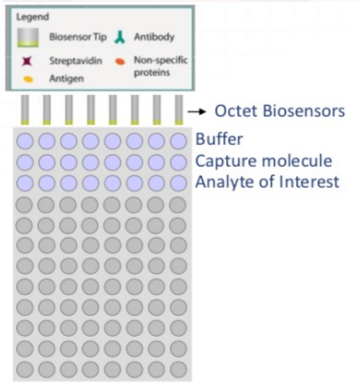
The FortéBio technology





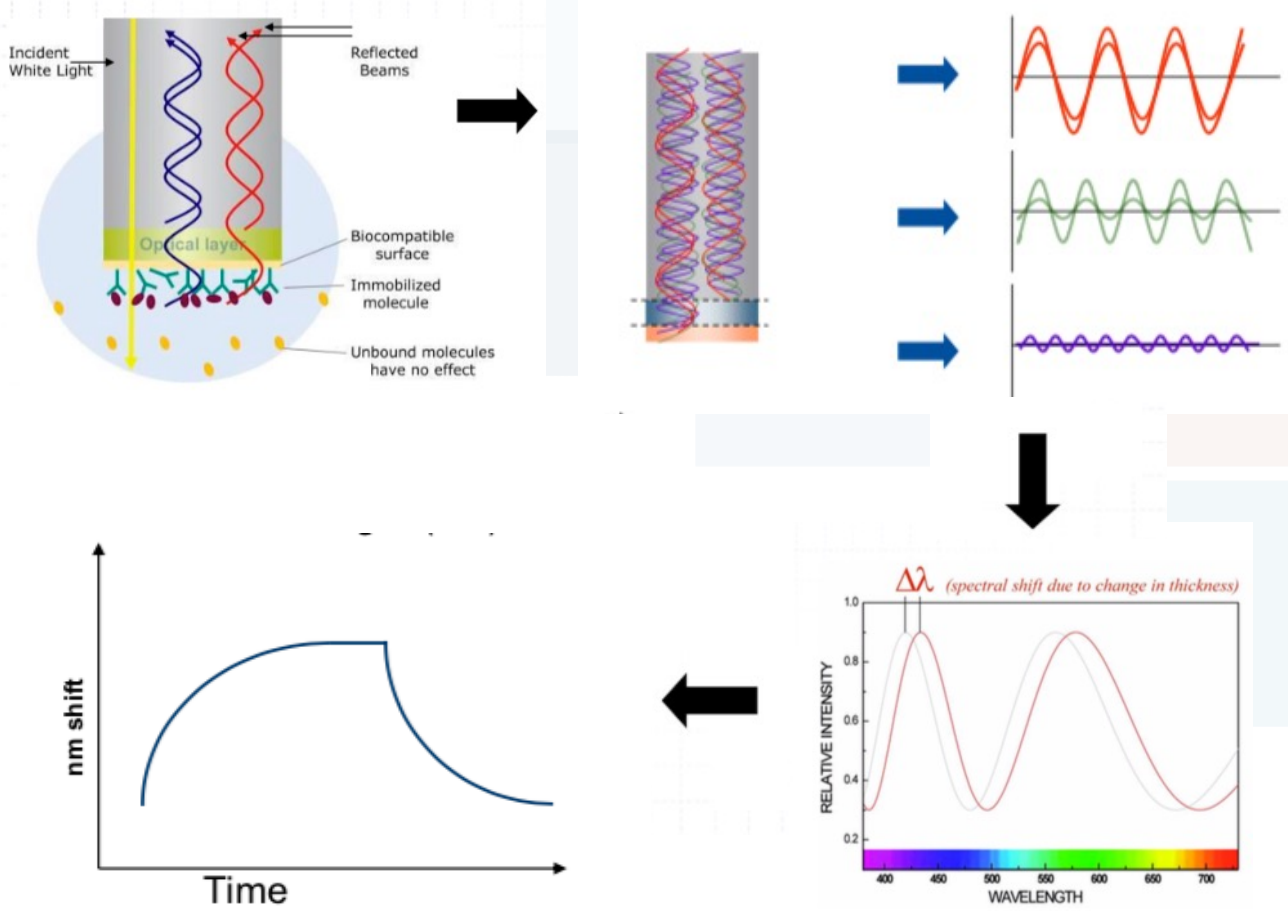






BLI

Basis of BLI measurements



- Bases on Reflectometric Interference Spectroscopy (RIfS) :
  - 1st optical layer reflects white light beam
  - 2nd biolayer reflects transmitted light
  - Both reach detector but with dephasing
  - Biomolecules binding changes "optical thickness" (n.d)
  - Spectral shift is measured

SPR/BLI

Pros & cons

Advantages:

- Label-free environment
- Real-time, continuous measurement
- Generic methods for diverse molecules
- Quick testing
- Small sample amounts & volumes
- Highly sensitive
- Specific to binding event
- Sensor chips can be regenerated
- Concentration in absence of a standard
- Or ELISA-like quantitation

Disadvantages

- Immobilization effects
- Steric hindrance with binding events
- Non-specific binding to surfaces
- Mass transport limitations
- Control experiments must be meticulously designed
- Misinterpretation of data common
- Expense of instrumentation, consumables (sensors)

Applications

- Wide range of interaction parameters

Specificity

YES/NO binding response

Concentration

How MUCH?

Affinity

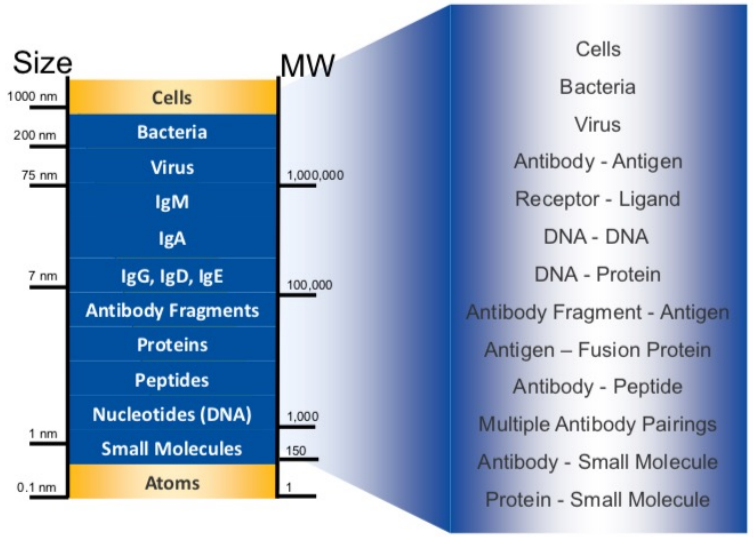
How STRONG? K_D, K_A

Kinetics

How FAST? Rate constants k_a, k_d

Thermodynamics

Affinity and kinetics *vs* temperature



- Large panel of applications for characterizing biomolecular interactions
- Wide range of molecules: from small molecules to cells
- Purified or crude samples
- Wide range of detection:
  - k_{on}: 10^1 to 10^7 $M^{-1}.s^{-1}$
  - k_{off}: 10^{-6} to 10^{-1} s^{-1}
  - K_D: 1 mM to 10 pM
  - Concentrations: 0.05 to 2000 µg/ml

SPR/BLI: introduction

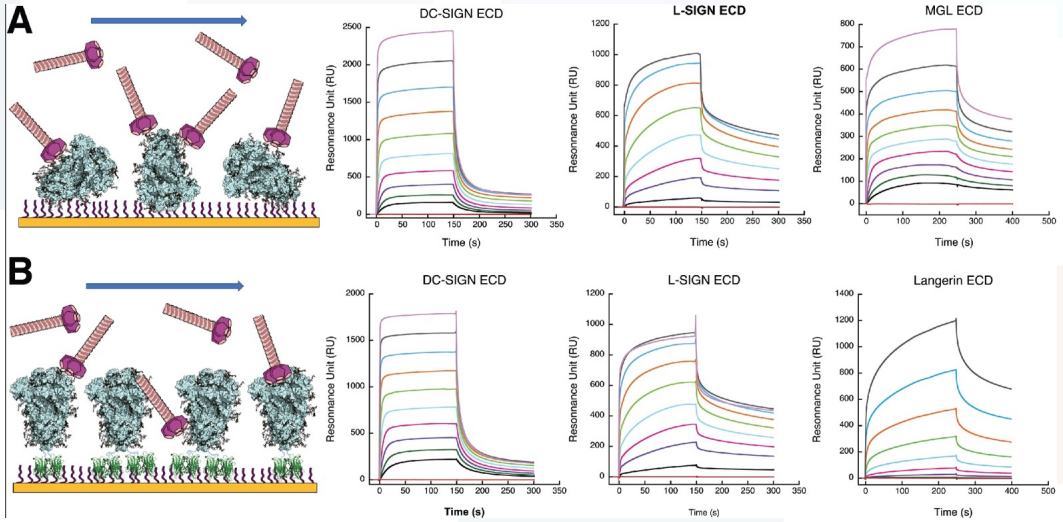
Objectives

- The SPR & BLI technologies for biomolecular interactions
- A few examples in Glycosciences
- A demonstration

A few examples

Immune Lectins-carbohydrate interactions

- By SPR
- Immobilized ligand: SARS-CoV-2 spike
- Soluble analytes: ectodomains of DC-SIGN, L-SIGN, MGL, Langerin



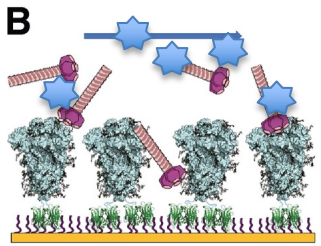
	DC-SIGN	L-SIGN	MGL	Langerin
	µM	µM	µM	
Non-oriented	11.90 ± 4.6	1.80 ± 0.12	7 ± 1.5	-
Oriented	4.33 ± 0.7	1.66 ± 0.12	-	n.d.

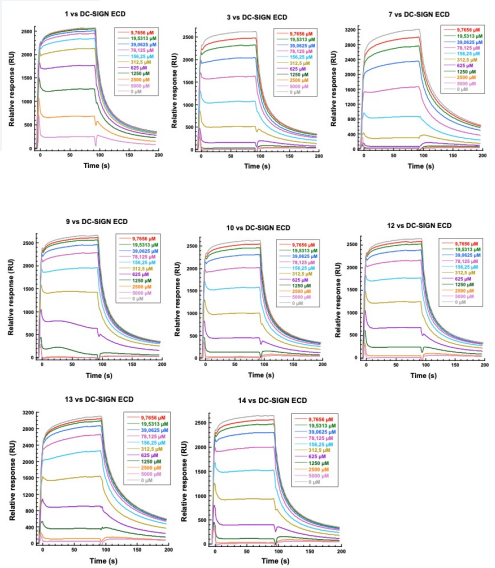
from Thépault et al. (2021) *PLOS Pathogens 1009576*

A few examples

Immune Lectins-carbohydrate inhibition

- By SPR
- Immobilized ligand: SARS-CoV-2 spike
- Soluble analytes: ectodomains of DC-SIGN or L-SIGN co-injected with glycomimetics
- Steady State responses => convertion lectin residual activity => EC50





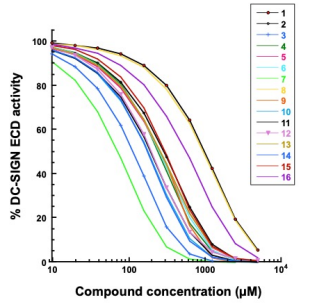
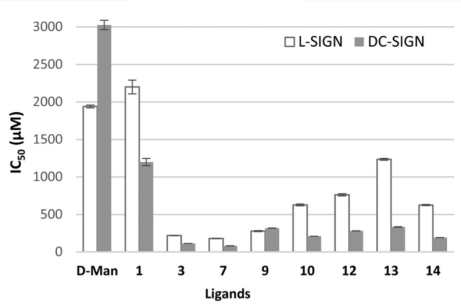


Figure SI-5 : Inhibition of DC-SIGN interaction with Man-BSA surface.



from Pollastri et al. (2022) *Chem. Comm. 58, 5136*

A few examples

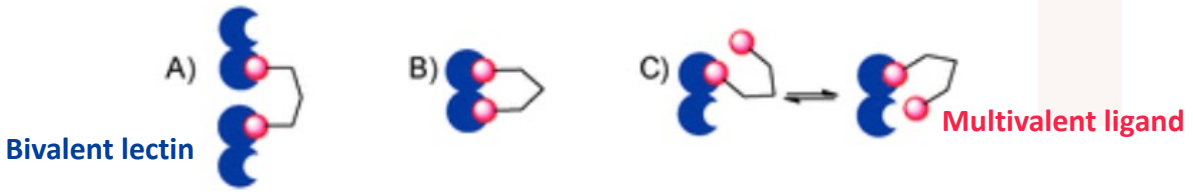
Lectin-carbohydrate interactions

- Sugar/protein interactions mainly involve multivalent partners

- **Affinity =** describes the binding of a monovalent ligand to its partner
- **Avidity =** takes into account multivalent interactions between partners

⇒ **Apparent enhanced functional affinity**

- Potency enhancement in multivalent ligands can result from different mechanism :

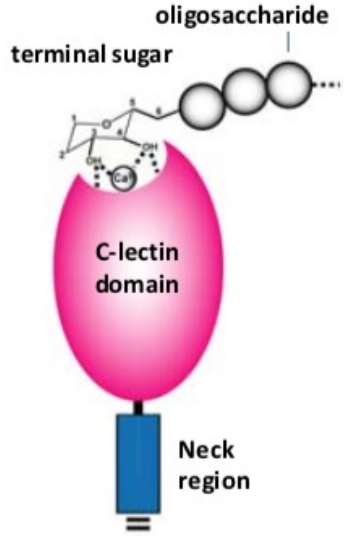


Adapted from Ordanini et al. (2015) *Chem. Commun. 51:3816*

Lectin-carbohydrate interactions

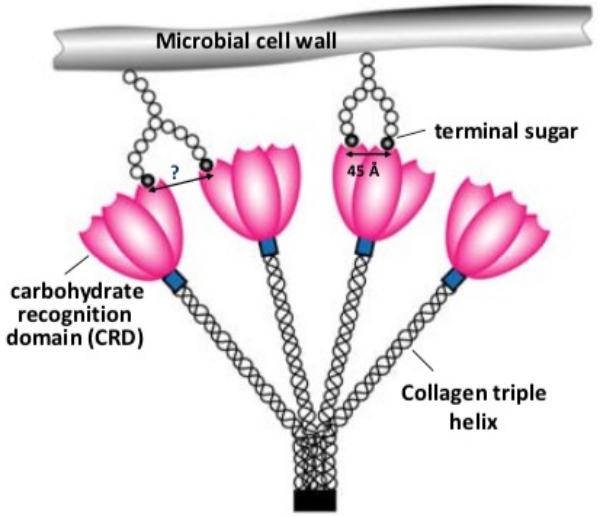
Mannan Binding Lectin (MBL)

Micropattern interaction



Low affinity ($K_D > 10^{-3}$ M)
1:1 binding

Macropattern interaction



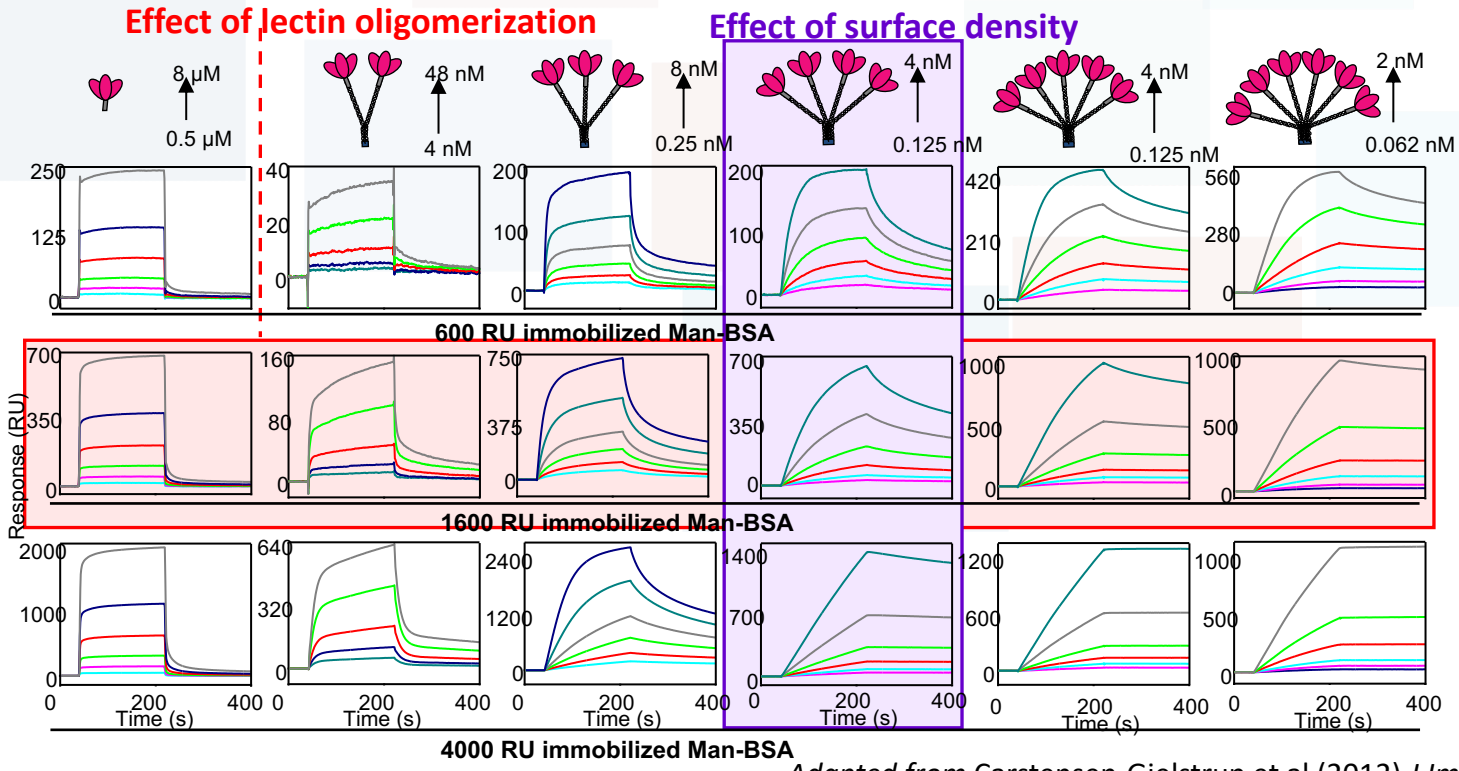
High affinity ($K_D = 10^{-9}$ M)
Multivalent binding

Adapted from Hoffmann et al. (1999) Sciences 284:1313

A few examples

Mannan Binding Lectin (MBL)

- Effect of avidity and surface density
  - Immobilized glycoconjugate (Man-BSA)
  - Soluble oligomeric lectin (MBL)



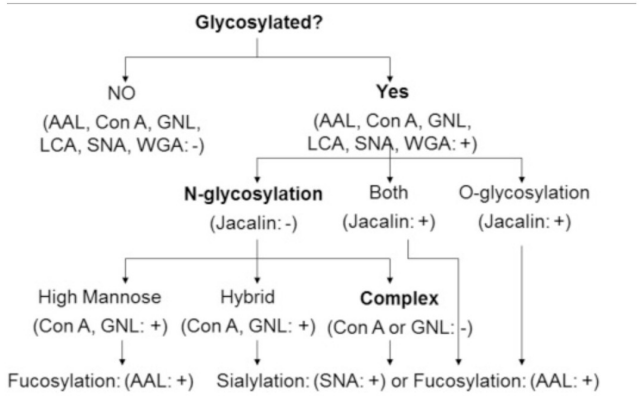
Adapted from Carstensen-Gjelstrup et al (2012) *J Immunol* 188:1292

A few examples

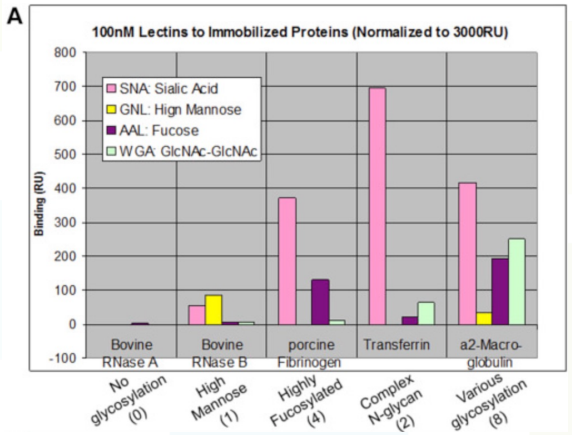
Glycan profiling

- By SPR
- Immobilized ligands: Proteins to profile
- Soluble analytes: lectins

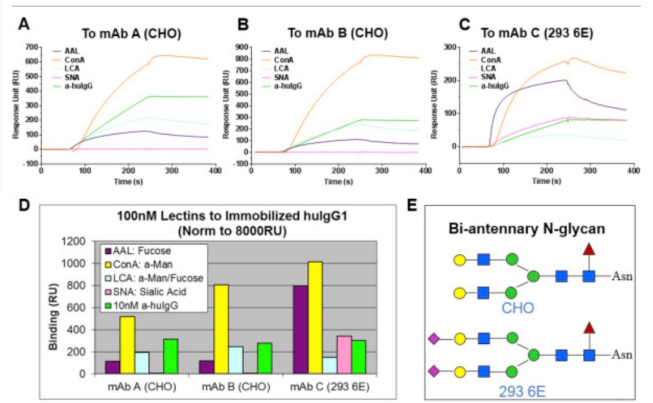
Lectin	Source	Specificity
AAL	*Aleuria Aurantia* mushrooms	Fucose
Con A	*Canavalia ensiformis* (Jack Bean) seeds	Mannose, Glucose
GNL	*Galanthus nivalis* (Snowdrop) bulbs	Mannose
jacalin	Artocarpus intergrifolia (Jackfruit) seeds	O-linked glycan
LCA	lentil seeds	Fucosylated mannose
SNA	*Sambucus nigra* (Elderberry) bark	Sialic Acid
WGA	*Triticum vulgaris* (wheat germ)	(GlcNAc)2, (GlcNAc)3, GlcNAc, Sialic Acid



Profile by comparing SPR signal levels



Between glycoproteins



Between recombinant expression systems

from Wang et al. (2017) *Annal. Biochem.. 538, 53-63*

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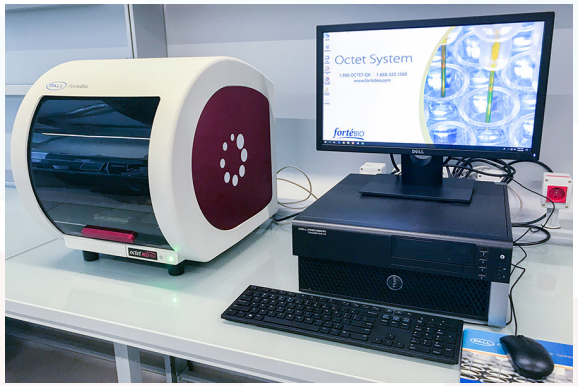
SPR/BLI

Institut de Biologie Structurale

Integrated Structural Biology Grenoble

SPR - Biacore T200 (Cytiva/Biacore)

BLI – OctetRED96e (Satorius/FortéBio)



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Surface Plasmon Resonance BioLayer Interferometry

"label-free" and real-time affinity & kinetics measurements

Jean-Baptiste REISER, Ph.D.

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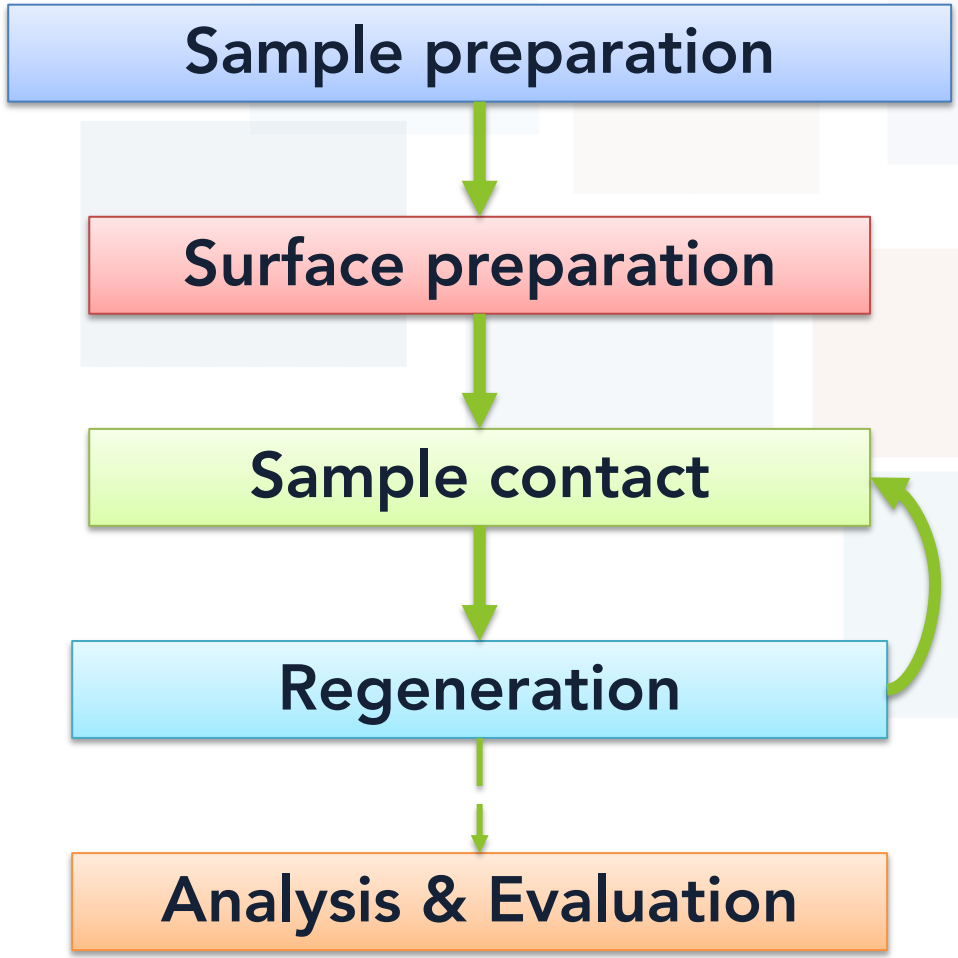
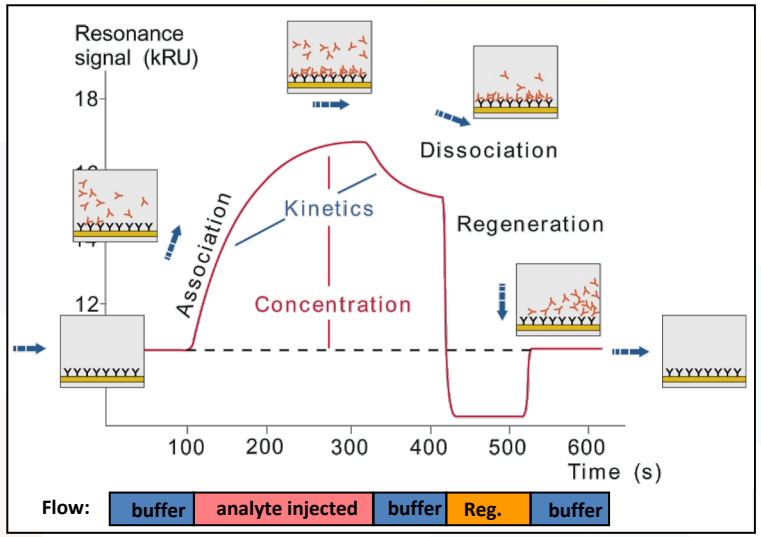
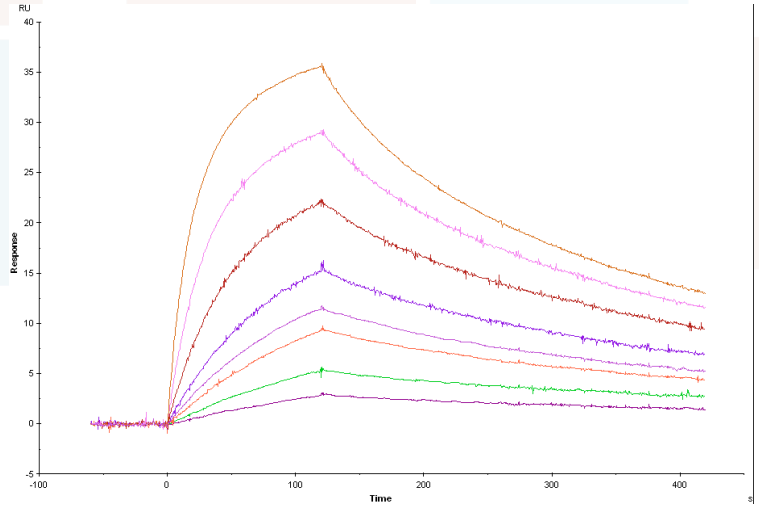
Introduction

Experimental design



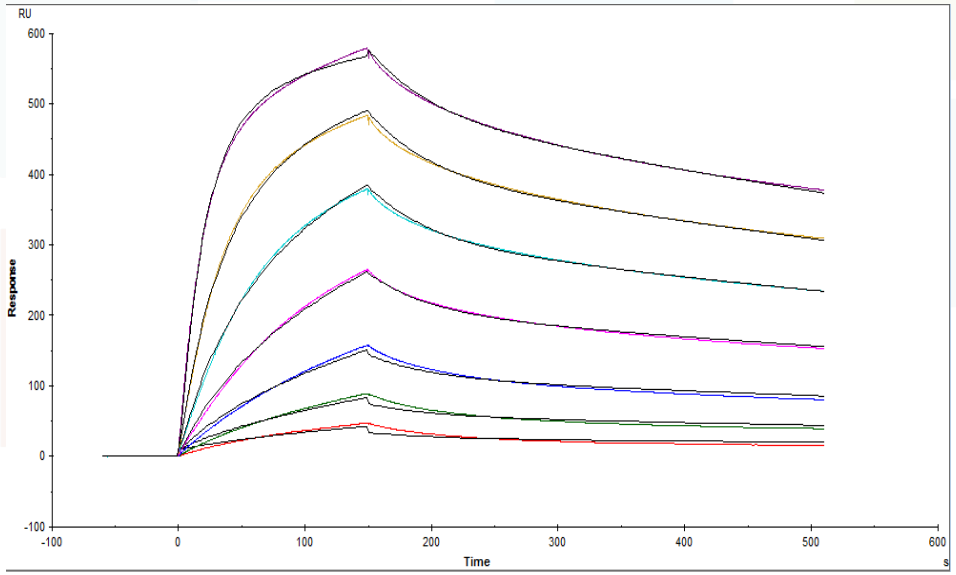




Practical aspects

Experimental design: Sample preparation & design

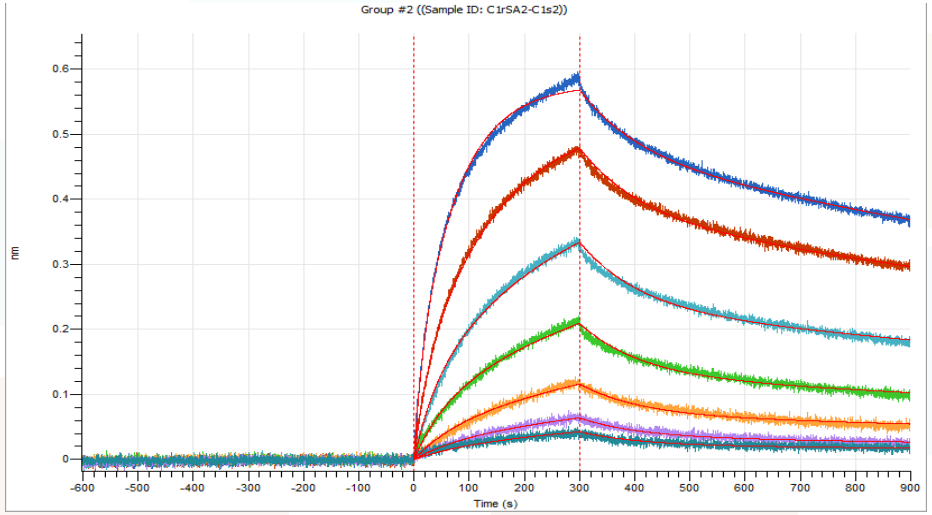
- Choice & purity of the reagents (biological samples & buffers)
- Ligand & analyte activities
- Ligand immobilization method (direct or indirect)
- Immobilization density
- Flow rate/shake speed
- Analyte concentration range
- Association and dissociation time ranges



Practical aspects

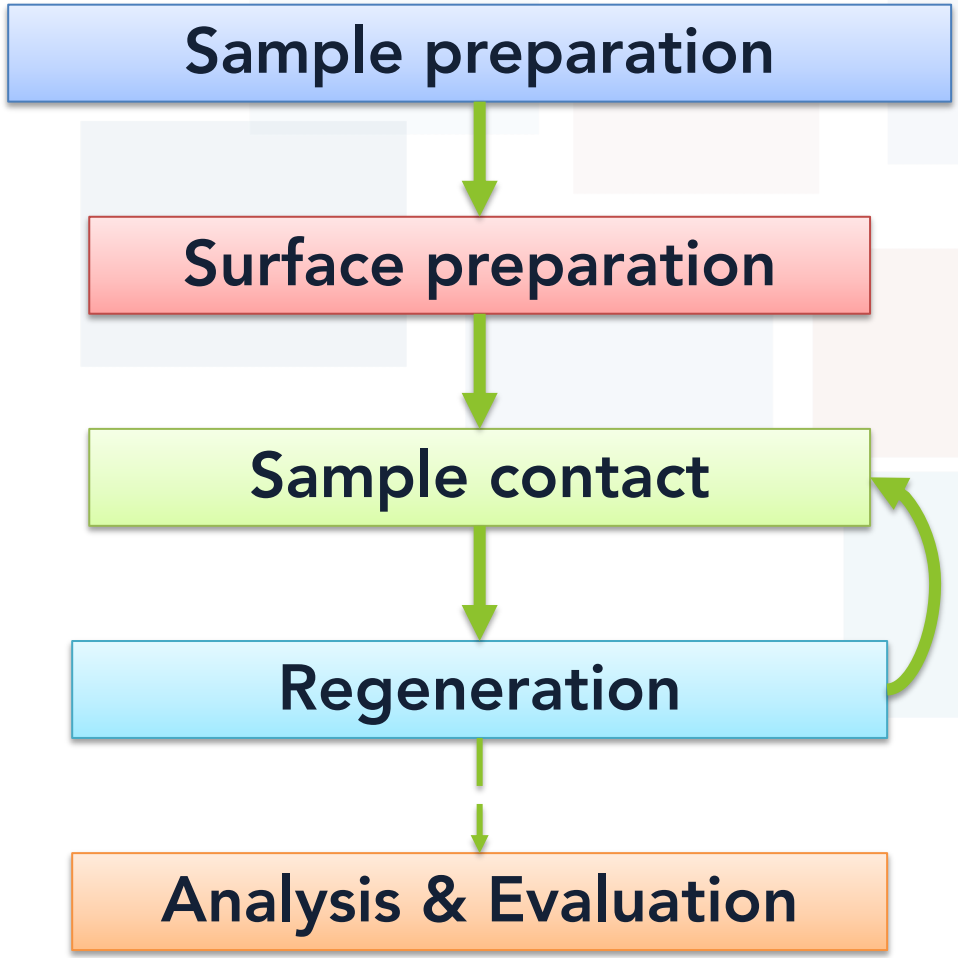
Experimental design: Sample preparation & design

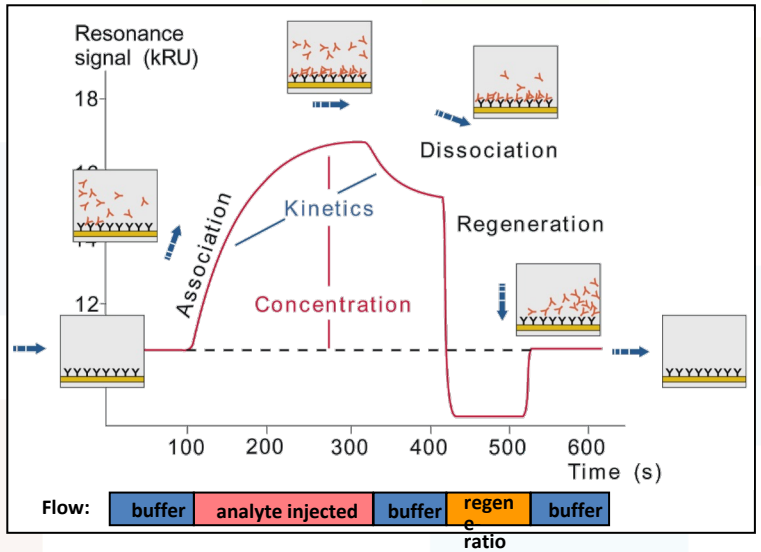
- Ligand preparation
  - High purity (if direct immobilization)
  - Concentration 10-200 µg/ml (1-20 µg)
  - Dilution in immobilization buffer (pH<pI-1 if amine coupling)
- Analyte preparation
  - Concentration determined accurately
  - High purity and homogeneity
  - Analyte buffer should match running buffer
  - Concentration range: 0.1*K_D to 10*K_D
  - Serial dilutions
- Running/Analysis buffer
  - Filtered (0.22 µm)
  - For SPR: Avoid unstable components (DTT) and high RI compounds (Glycerol, DMSO)
  - Additives to avoid non-specific binding to sensors: Detergent P20/Tween-20 (0.005 to 0.2%) – BSA (0.01 – 0.1%) – others (dextran, biocytine…)

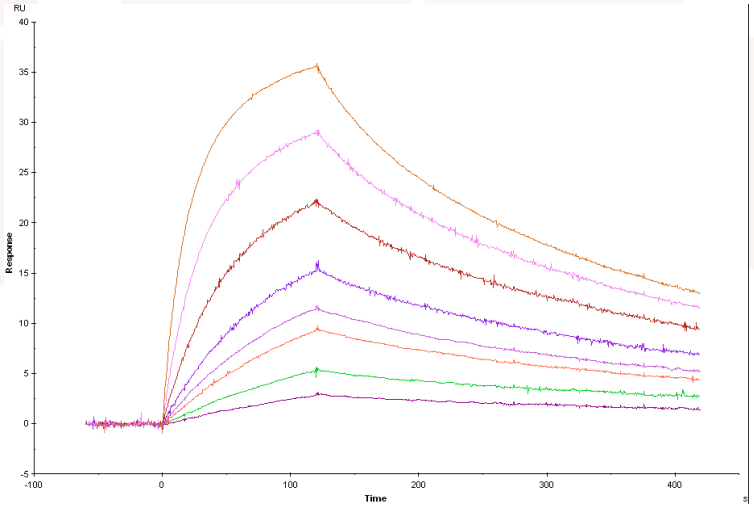


Practical aspects

Experimental design



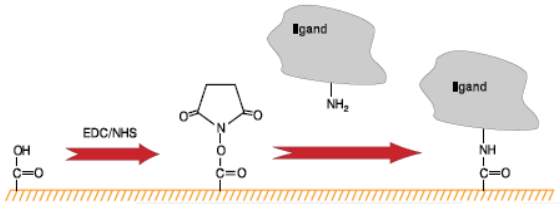




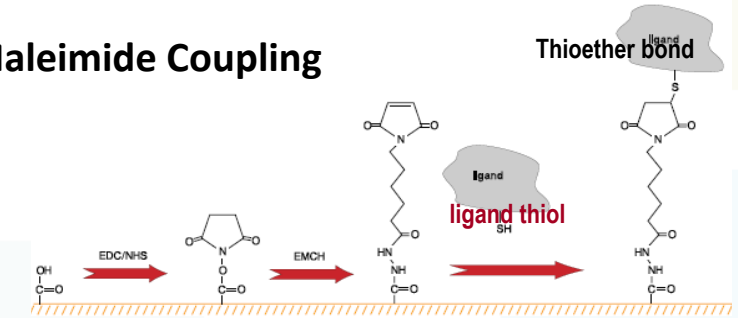
Practical aspects

Experimental design: Immobilization strategy

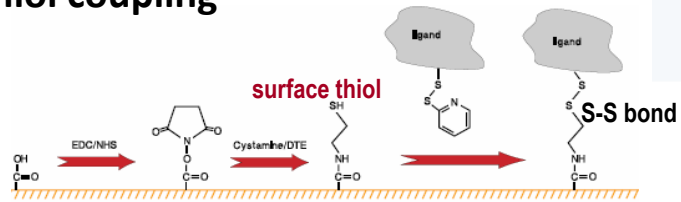
Amine coupling



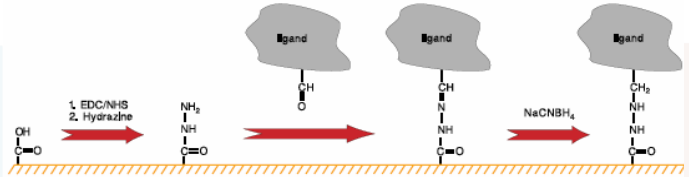
Maleimide Coupling



Thiol coupling



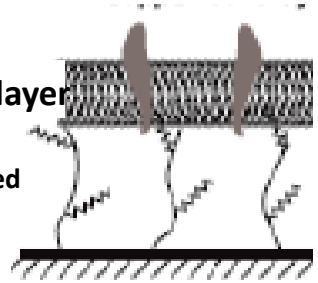
Aldehyde coupling



Integral membrane proteins (L1 chip)

Supported bilayer

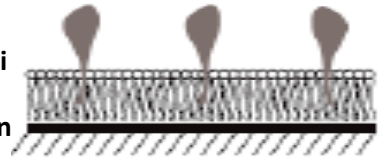
CM dextran modified with lipophilic compounds



Membrane-bound proteins (HPA chip)

Adsorbed lipid monolayer

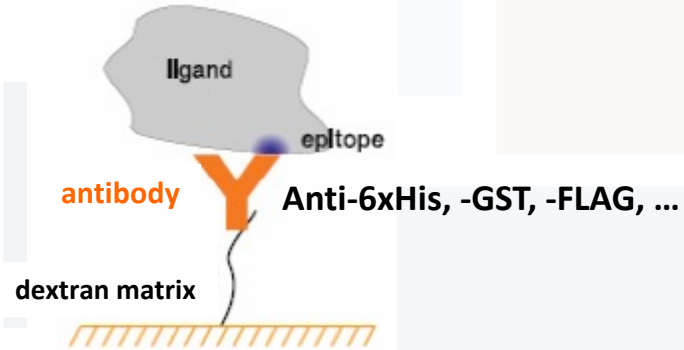
Hydrophobic surface no dextran



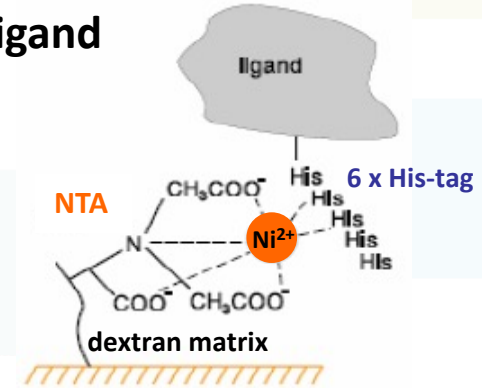
Practical aspects

Experimental design: Immobilization strategy

Antibodies

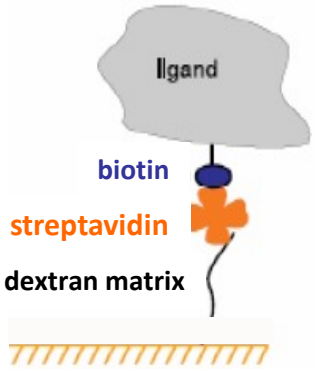


His-tagged ligand

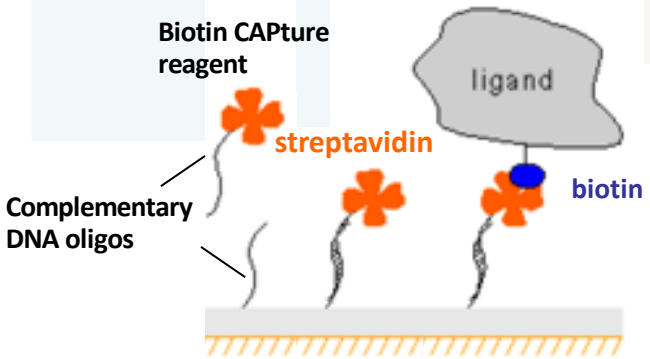


NTA chip

Biotinylated ligand



SA chip



CAP chip

Practical aspects

Experimental design: Immobilization strategy

SPR sensorchips

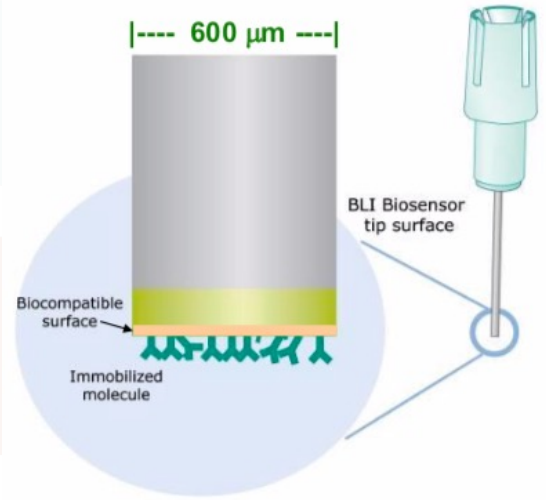
	Surface type	Uses
CM5	CM-dextran (100 nm)	general purpose
CM4	same as CM5, lower carboxylation degree	low immobilization levels and reduced non-specific binding
CM3	same as CM5, shorter dextran (40 nm)	large ligand molecules and particles
CM7	same as CM5, higher carboxylation degree	studies with small molecules and fragments
SA (CAP)	CM-dextran with immobilized streptavidin	(reversible) capture of biotinylated ligands
Protein A	CM-dextran with immobilized recombinant Protein A variant	oriented capture or binding of antibodies through Fc region only
NTA	CM-dextran with immobilized NTA	capture of polyHis-tagged ligands
L1	CM-dextran with lipophilic modification	capture of liposomes
HPA	Flat hydrophobic surface, no dextran matrix	capture of lipid monolayers
C1	Flat carboxylated surface, no dextran matrix	applications where dextran is undesirable
Au	Plain gold surface	user design of surface chemistry
SIA kit	Plain gold surface, not mounted on chip support	surface modifications outside Biacore systems

Practical aspects

Experimental design: Immobilization strategy

Biosensor	Application
Antibody-Specific Capture	
• Anti-Human IgG Fc Capture (AHC)	Human IgG Fc region, kinetic analysis
• Anti-Human IgG Fc Capture (AHQ)	Human IgG Fc region, quantitation
• Anti-Mouse Fc Capture (AMC)	Mouse IgG1, 2a & 2b Fc regions, kinetic analysis
• Anti-Mouse Fc Capture (AMQ)	Mouse IgG1, 2a & 2b Fc regions, quantitation
• Anti-Human Fab-CHI (FAB)	Fab-CH1 domains of human IgG
• Protein A (ProA)	Quantitation of various species IgG
• Protein G (ProG)	Quantitation of various species IgG
• Protein L (ProL)	Quantitation of IgG via kappa light chain
Affinity Tag Capture	
• Streptavidin (SA)	Biotinylated ligands
• High Precision Streptavidin (SAX)	Biotinylated ligands (enhanced precision)
• Super Streptavidin (SSA)	Biotinylated ligands (high-density surface)
• Anti-GST (GST)	GST-tagged recombinant proteins
• Anti-Penta HIS (HIS1K)	HIS-tagged recombinant proteins
• Anti-Penta HIS 2nd Gen (HIS2)	HIS-tagged recombinant proteins
• Ni-NTA (NTA)	HIS-tagged recombinant proteins
Immobilization	
• Amine Reactive 2nd Gen (AR2G)	Covalent coupling to reactive amine groups
• Aminopropylsilane (APS)	Adsorption to hydrophobic moieties

BLI biosensors



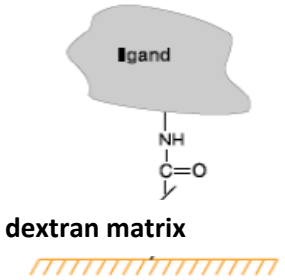
SPR : kinetic & affinity analysis

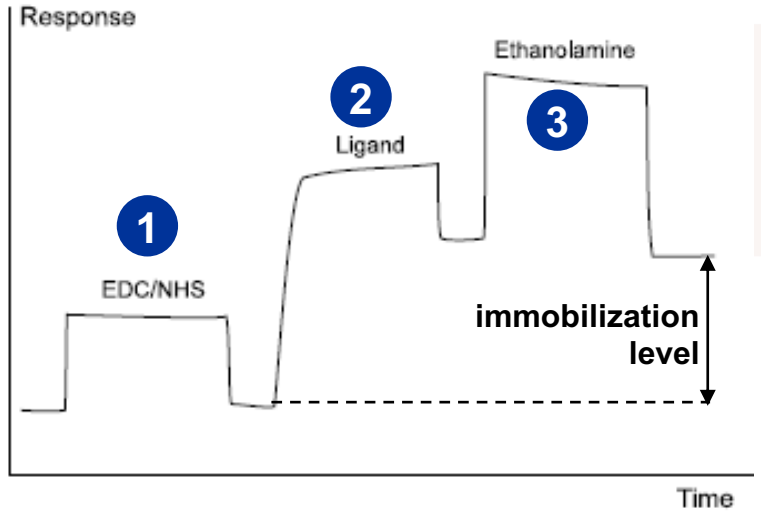
Experimental design: Surface preparation

- Typical covalent amine coupling
- Avoid Tris buffer (primary amine)
- pH of dilution buffer 0.5 to 1 unit below the ligand pI
- Low ionic strength (10-15 mM max)
- Immobilization level ?

$$R_{ligand} = R_{max} \cdot \frac{MW_{ligand}}{MW_{analyte}} \cdot \frac{1}{V} \cdot \%_{active\ ligand}$$

1. Activation of carboxymethylated dextran matrix by forming reactive succinimide ester
2. Coupling through primary amine groups
3. Saturation of remaining active CM groups with Ethanolamine





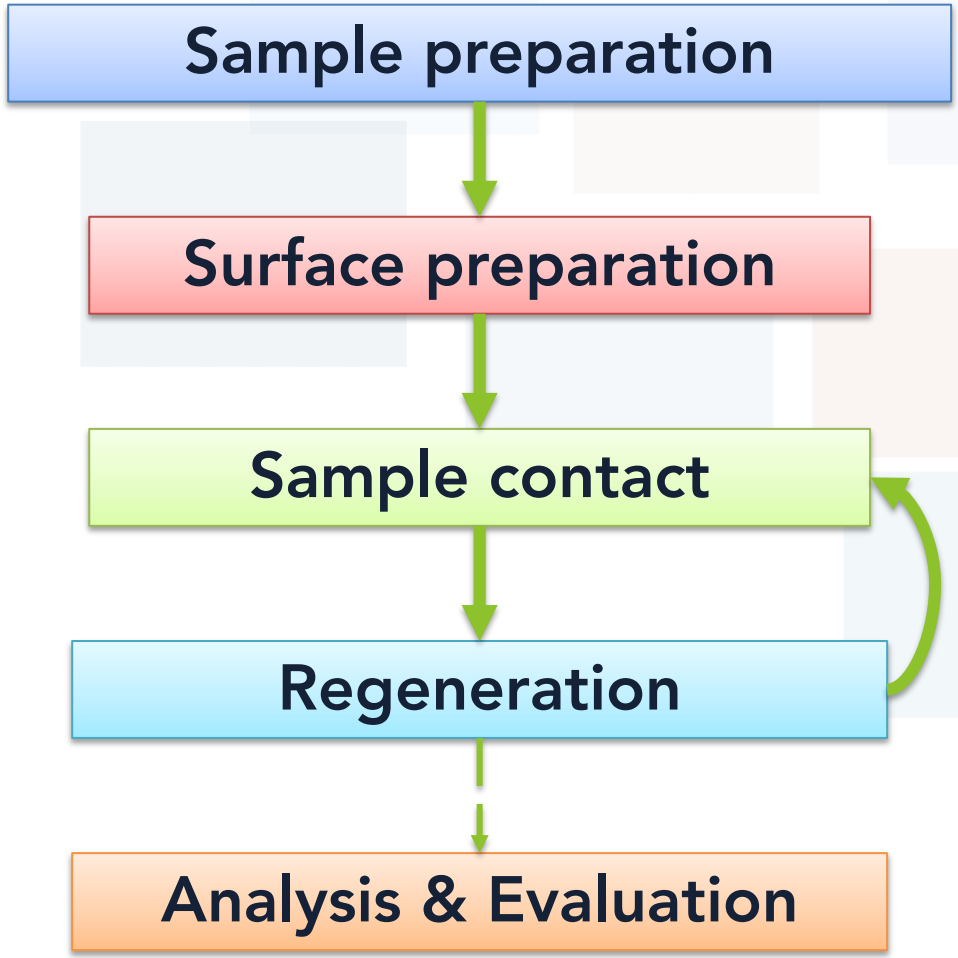
Practical aspects

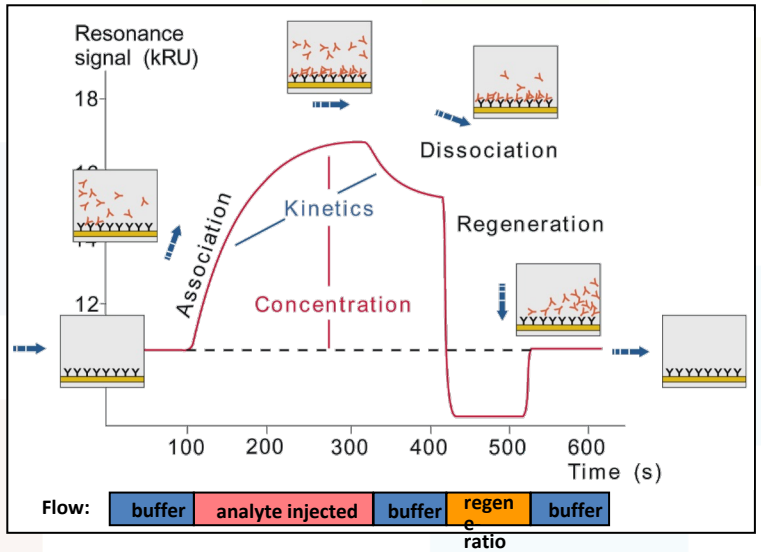
Experimental design: Surface preparation

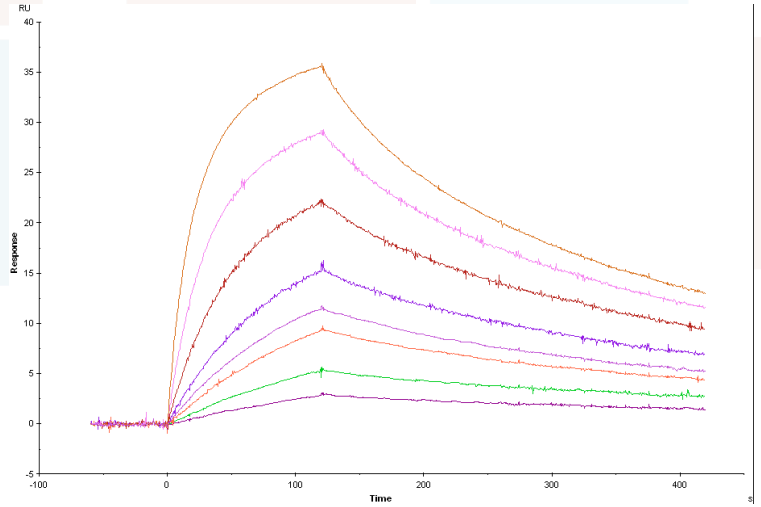
- Non specific analyte/surface interaction
- Bulk contribution due to change in buffer composition between running and sample buffers. Can be minimized with using same buffer.
- Baseline drift
- Need of a reference surface
  - Unmodified surface:
    - acceptable in many cases
  - Activated-Saturated surface:
    - treating the surface with same procedure but omitting the ligand
  - Dummy ligand:
    - immobilization of an irrelevant ligand (scrambled peptide, BSA, inactive mutant…) to mimic the active surface as closely as possible

Practical aspects

Experimental design



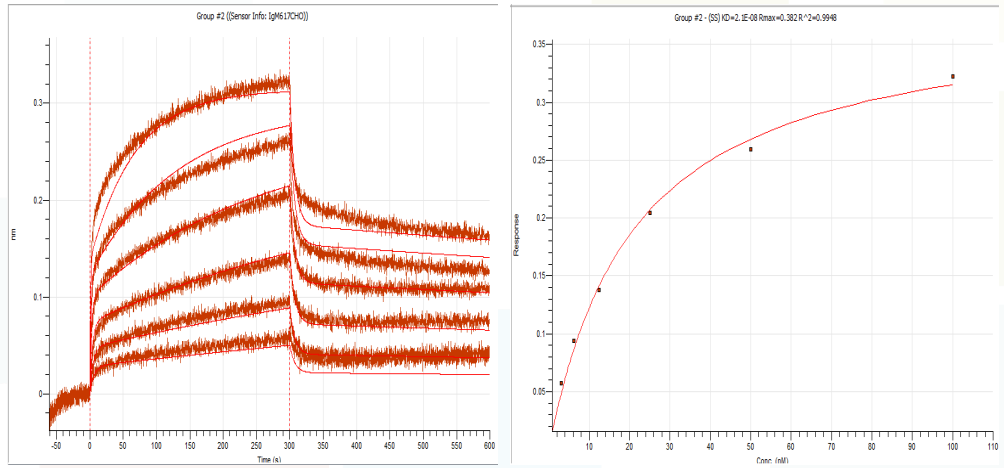




Practical aspects

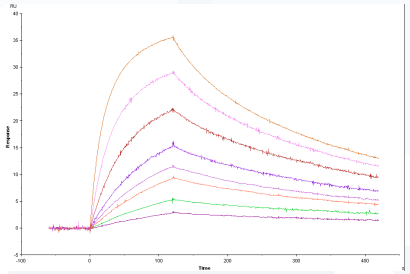
Experimental design: important considerations

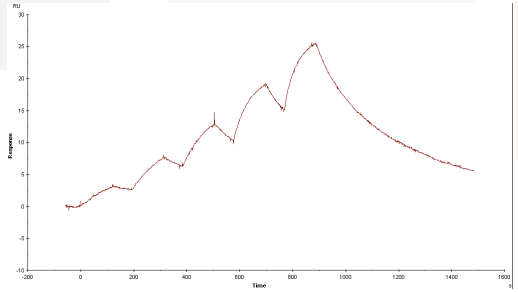
- Analyte concentration: 0.1 to 10 times the estimated K_D
- Minimum of 5 concentrations
  - Dilution in series
  - Injections from low to high concentrations
- 1 zero-concentration sample
  - i.e. buffer
  - for double reference
- 1 concentration in duplicate to assess reproducibility
- Choose flow rate/shake speed to minimize mass transfer effects
- Adjust association time to reach equilibrium
  - Dependent of k_a, k_d, C
- Adjust dissociation time to observe at least 5% of dissociated analyte
  - $t_{5\%} = 0.052/k_d$
- Replicate your experiment

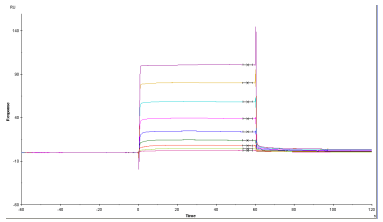


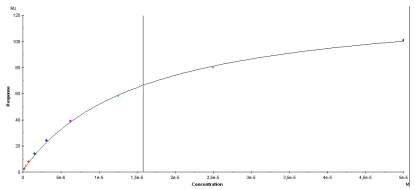
SPR : kinetic & affinity analysis

Experimental design: types of analyses





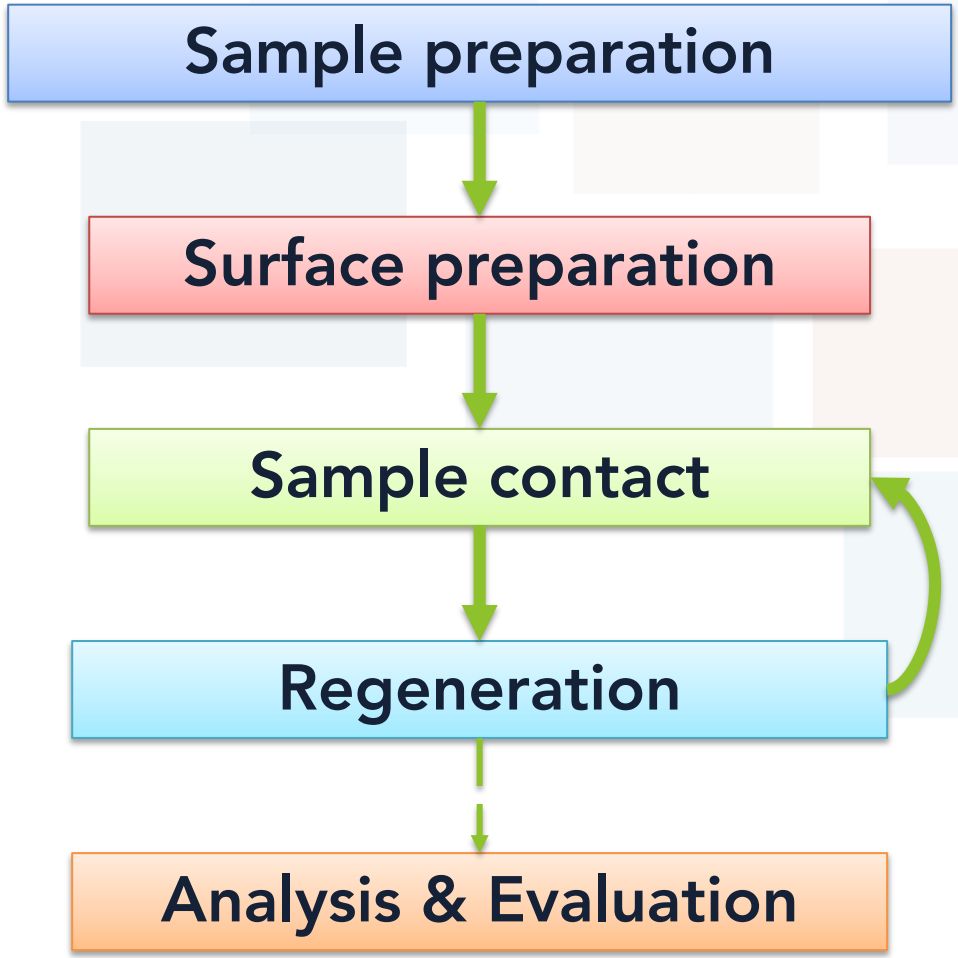


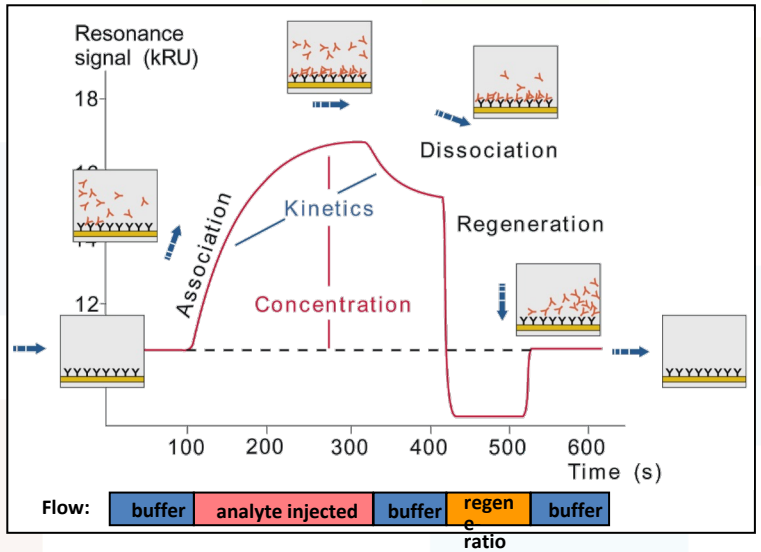


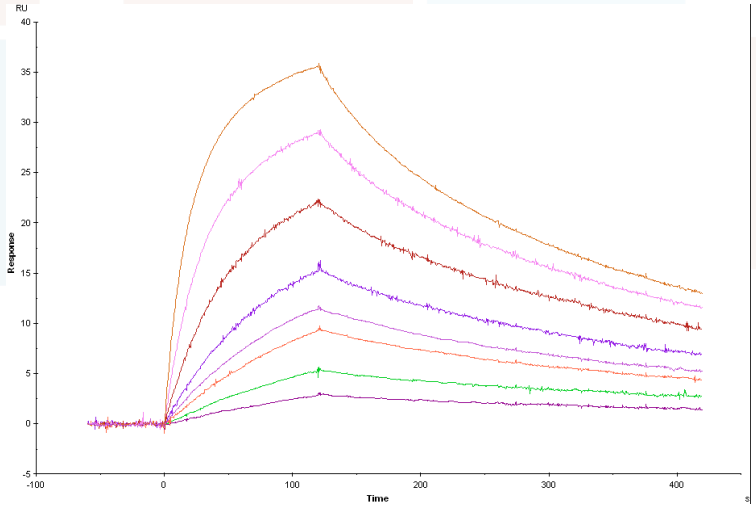
- Multi-cycle kinetics:
  - 5 to 10 analyte concentrations
  - Regeneration may be needed
  - Monitor association and dissociation rates (K_D, k_d, k_a)
- Single-cycle kinetics:
  - 5 analyte concentrations in series
  - Regeneration not needed
  - Monitor association and dissociation rates (K_D, k_d, k_a)
- Steady state:
  - 5 to 10 analyte concentrations
  - Regeneration may be needed
  - Monitor only K_D

Practical aspects

Experimental design



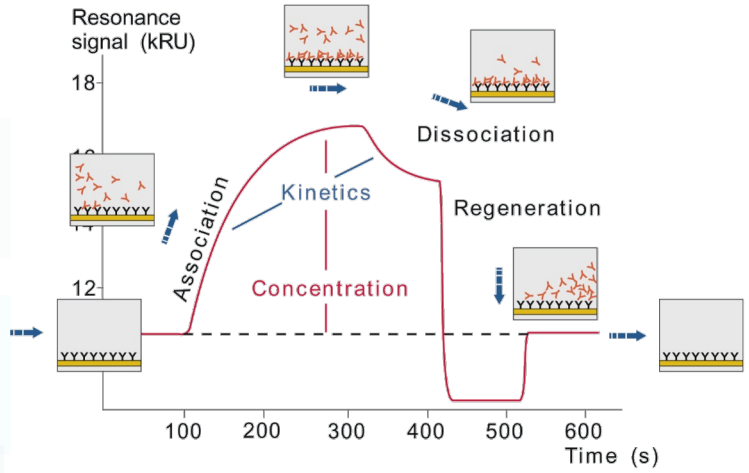




Practical aspects

Experimental design: Regeneration

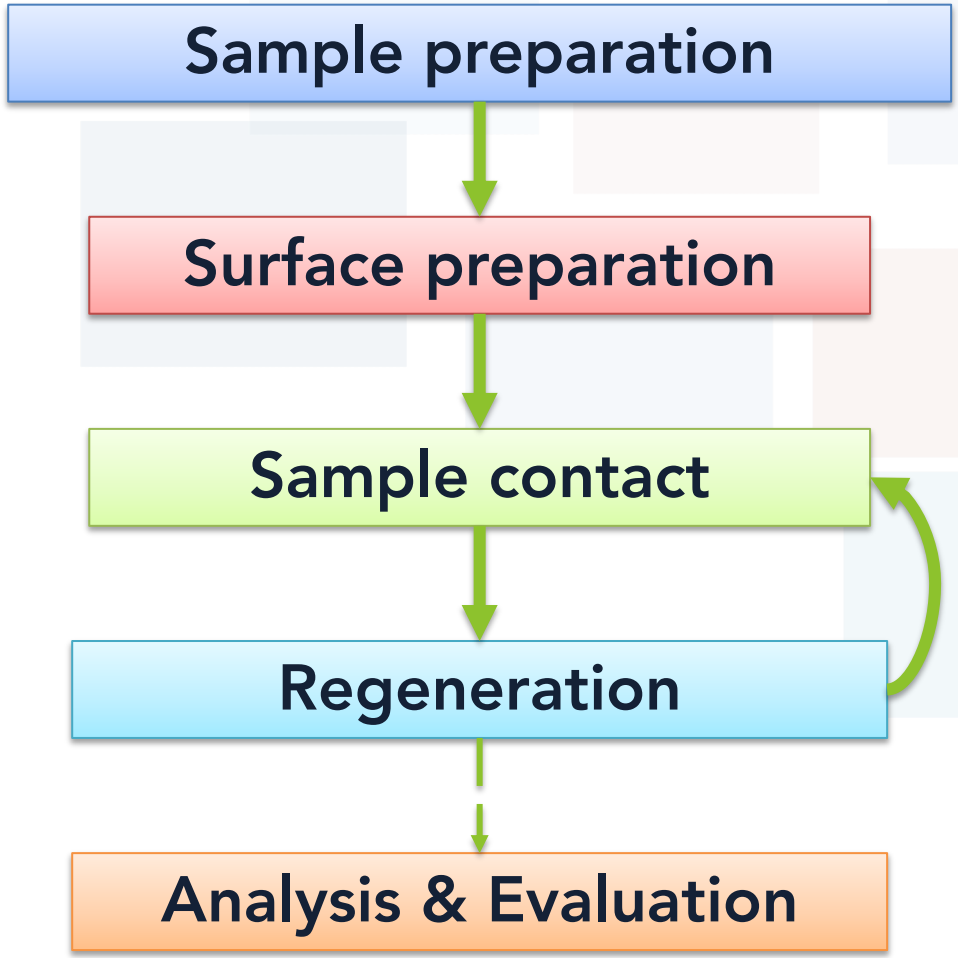
- Necessary between cycles if the dissociation is to slow
- To remove bound analyte completely from the surfaces
- Efficient regeneration is crucial for high quality data
  - The activity of the immobilized ligand must remain unaffected
  - Regeneration solution selected according to the nature of the interaction (if known, specific competitor, high salt concentrations, pH variations…)

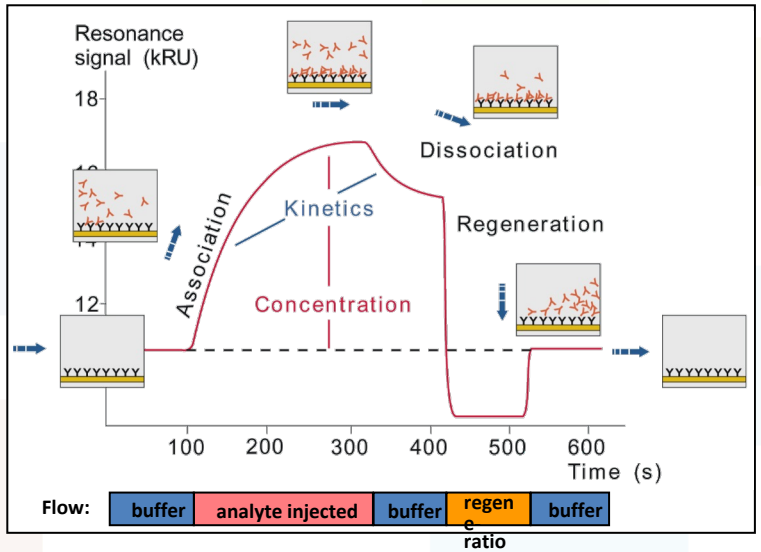


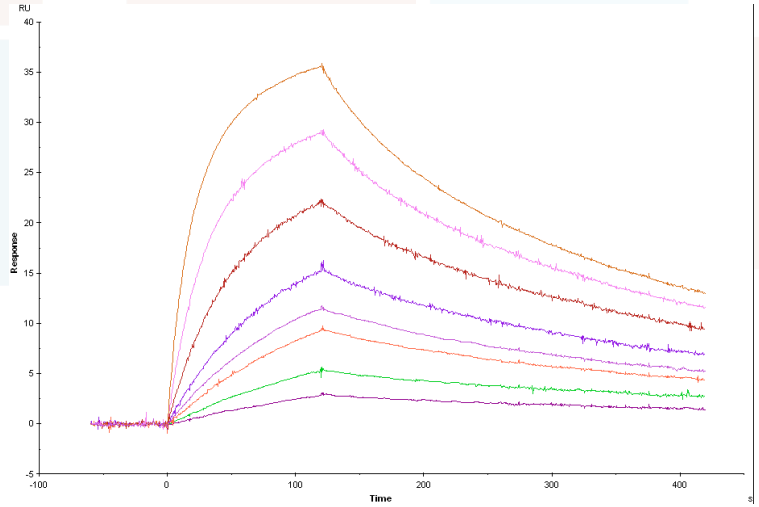
Strength	Acidic	Basic	Hydrophobic	ionic
Weak	pH > 2.5 formic acid, HCl 10 mM Gly/HCl	pH < 9 10 mM Hepes/NaOH	pH < 9 50% ethylene glycol	1 M NaCl
Intermediate	pH 2- 2.5 formic acid, HCl 10 mM Gly/HCl H_3PO_4	pH 9-10 NaOH 10 mM Gly/NaOH	pH 9-10 50% ethylene glycol	2 M $MgCl_2$
Strong	pH < 2 formic acid, HCl 10 mM Gly/HCl H_3PO_4	pH > 10 NaOH	pH > 10 25-50% ethylene glycol	4 M $MgCl_2$ 6 M GdnCl

Practical aspects

Experimental design





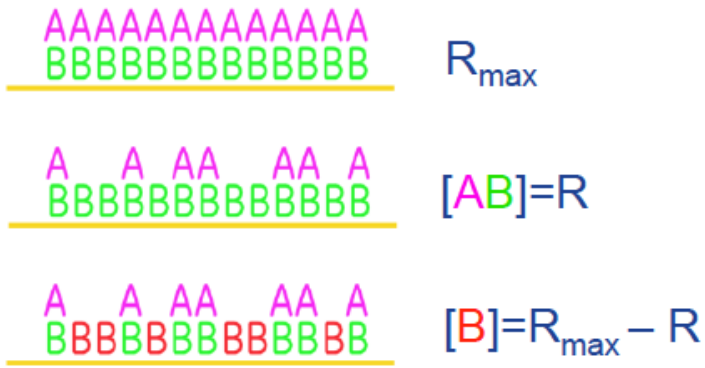


Practical aspects

Data handling and processing

$$A + B \underset{k_d}{\overset{k_a}{\rightleftarrows}} AB$$

- A is the analyte in solution:
  - Its concentration (C) is maintained by constant flow
- AB is the complex:
  - Its concentration is measured by R
  - R_{max} is the maximal feasible response
- B is the ligand on the surface:
  - Concentration of free ligand is derived from R_{max}-R



$$\text{net rate: } \frac{d[AB]}{dt} = k_a.[A].[B] - k_d.[AB]$$

$$\text{net rate in SPR: } \frac{dR}{dt} = k_a.C.[R_{max}-R] - k_d.[R]$$

Practical aspects

Data handling and processing

- Classical 1:1 langmuir absorption surface:
  - Can be integrated:

$$R = R_{eq}.(1-e^{-(k_a.C+k_d).(t-t_0)})$$

  - More complex models (mass transfer, 2 steps reaction…) are not
    - Numerical integration (non-linear regression)
    - Global analysis of several data sets

- Non-linear regression:
  - Find values of variables, which relate Y to X, giving the closest fit and reducing the residual

$$Chi^2 = \frac{\sum (R_{fit}-R_x)^2}{n-p}$$

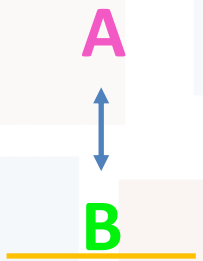
  - Iterative process, convergence criteria, local minimum values

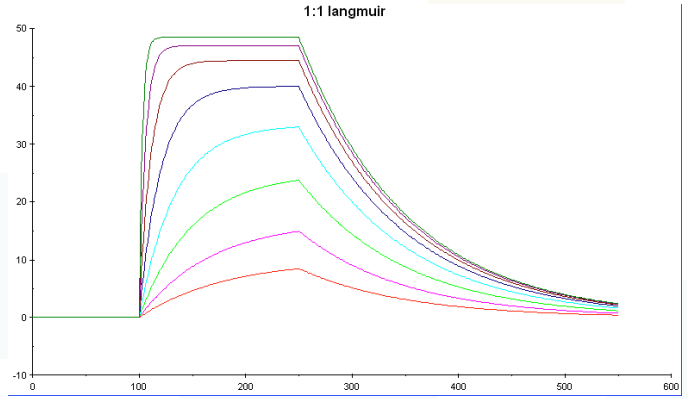
Practical aspects

Data handling and processing: kinetics models

- 1:1 langmuir

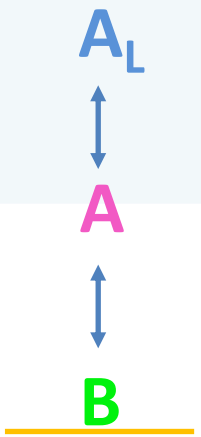
$$A + B \underset{k_d}{\overset{k_a}{\rightleftarrows}} AB$$

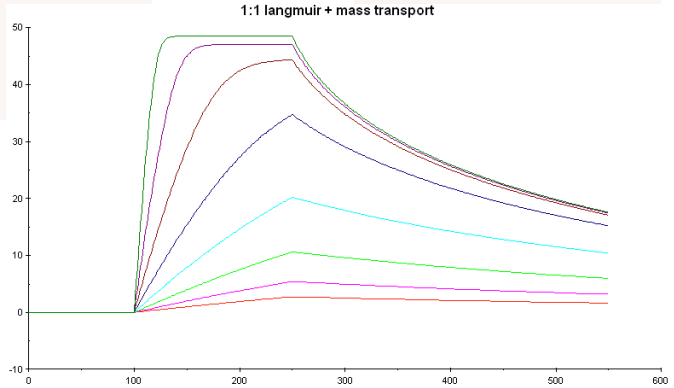




- 1:1 langmuir + mass transfer

$$A_{sol} \underset{}{\overset{k_t}{\rightleftarrows}} A_{surf} + B \underset{k_d}{\overset{k_a}{\rightleftarrows}} A_{surf}B$$



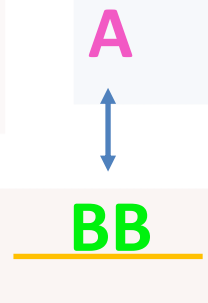


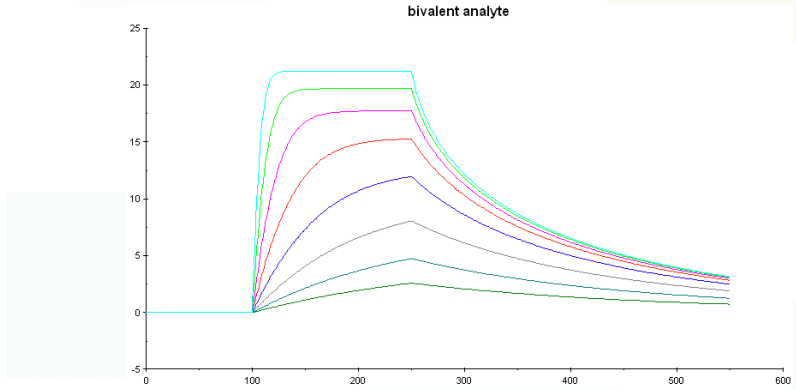
Practical aspects

Data handling and processing: kinetics models

- Bivalent analyte

$$A + 2B \underset{k_{d1}}{\overset{k_{a1}}{\rightleftarrows}} A + AB \underset{k_{d2}}{\overset{k_{a2}}{\rightleftarrows}} A2B$$



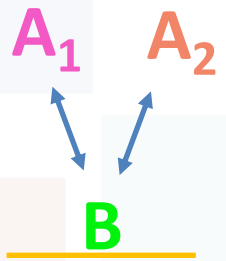


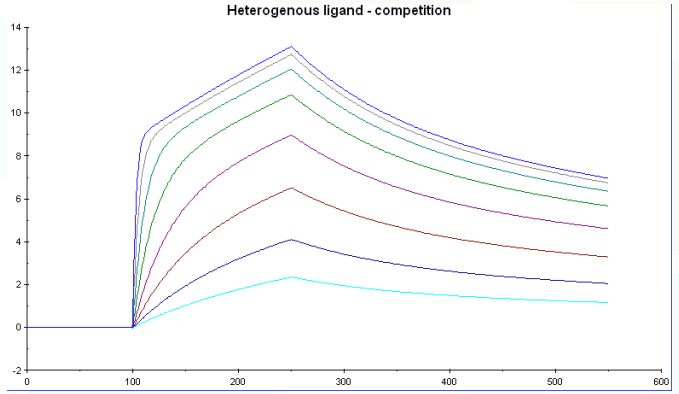
Practical aspects

Data handling and processing: kinetics models

- Heterogenous analyte *(competition)*

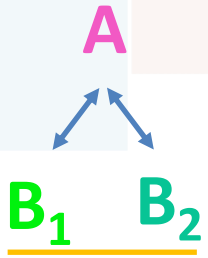
$$A_1 + B \underset{k_{d1}}{\overset{k_{a1}}{\rightleftarrows}} A_1B \;;\; A_2 + B \underset{k_{d2}}{\overset{k_{a2}}{\rightleftarrows}} A_2B$$

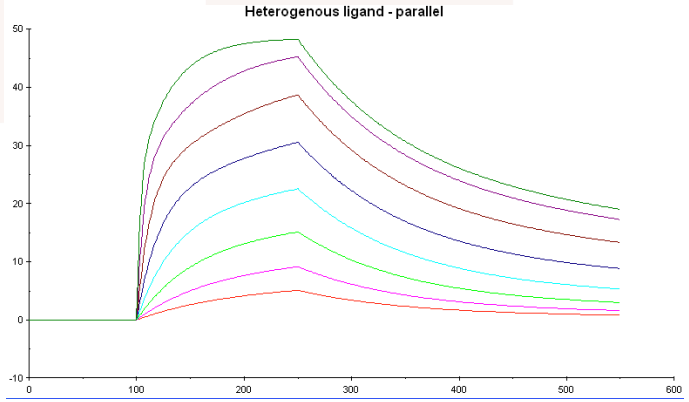




- Heterogenous ligand *(parallel reaction)*

$$A_1 + B \underset{k_{d1}}{\overset{k_{a1}}{\rightleftarrows}} A_1B \;;\; A_2 + B \underset{k_{d2}}{\overset{k_{a2}}{\rightleftarrows}} A_2B$$





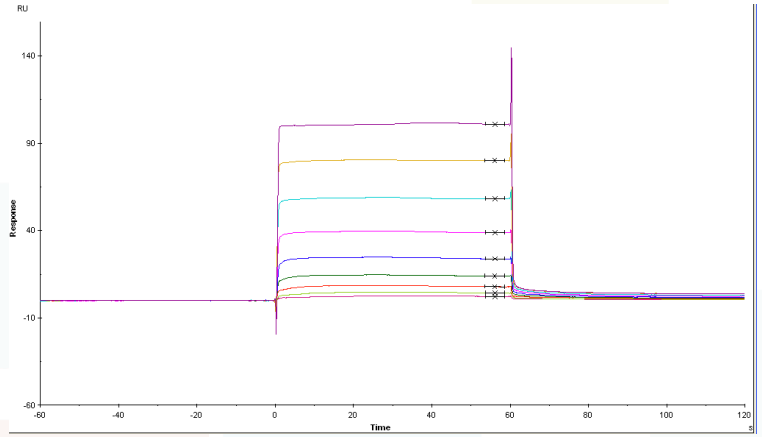
Practical aspects

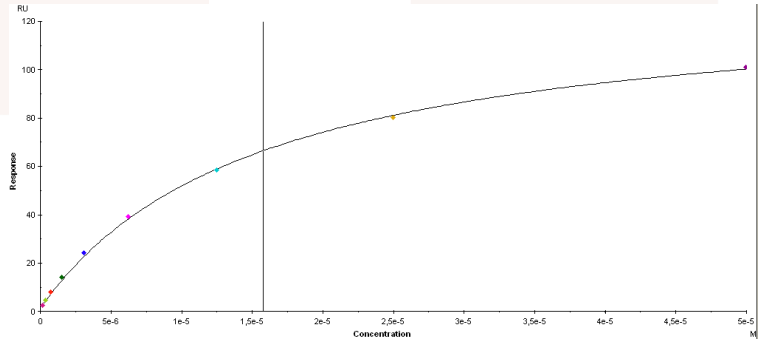
Data handling and processing: Steady-State model (affinity only)

- Steady State/equilibrium analysis
  - Only when steady state reached
  - Plot of equilibrium response (R_{eq}) vs. Analyte Concentration (C)

$$R_{eq}(C) = \frac{C.R_{max}}{K_D + R_{max}}$$

$$R_{eq}(C=K_D) = {}^{R_{max}}/_{2}$$





Practical aspects

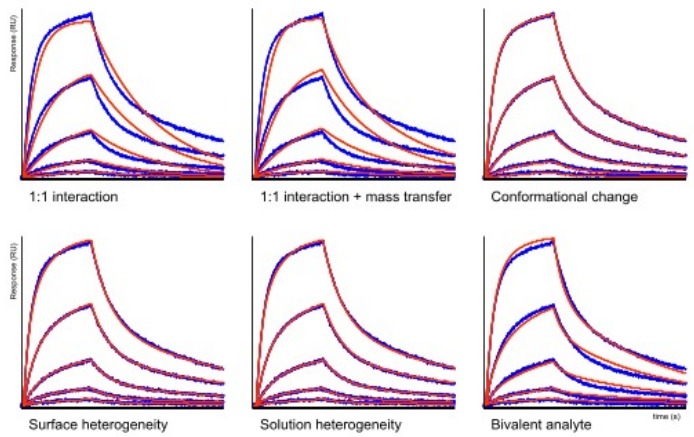
Quality of the evaluation/Data fitting

- *Magnitude of kinetics constants*: within the instrument range?
- *Parameter uniqueness*: does correlation between fitted variables exist ? If yes: parameters are linked during fitting (U value)
- *RI*: as low as possible. If experience well designed, RI must be minimum
- *Sensorgram curvatures*
- *Residuals*: as low as possible. Should be in the same order of magnitude as the instrument noise (1-5 RU)
- *Chi*^{2}: should be in the same order of magnitude as the instrument noise. But it is dependents of the number of measurments and of magnitude of the measurements.
- *Stantard errors*: calculated SE from fitting should be <10%
- *Rmax* < Theorical Rmax
- *Self consistancy*

Practical aspects

Quality of the evaluation/Data fitting

- Careful : model surfing/shopping is not a good solution. More variables in the model always lead to better data fitting statistics.
- *Use of a model must be motivated. Use the simplest model*
- *Other validation tips:*
  - *Validate your experimental settings (quality of samples, immobilization levels, association, dissociation times, concentration ranges, blank injections…)*
  - *Repeat the experiments (mutiplicates)*
  - *Use different immobilization levels*
  - *Vary flow rates (mass transport)*
  - *Vary analyte contact time*
  - Check self consistancy (kinetics, equilibrium analysis)



Practical aspects

What must be published in M&M:

- Formalizing reporting: Minimum information about a protein affinity reagent (MIAPAR, Bourbeillon *et al.*, 2010).
- From Myszka *et al.*: TBMRFAADOBE = The Bare Minimum Requirements For An Article Describing Optical Biosensor Experiments

instrument used in analysis
identity, source, molecular weight of ligand and analyte
surface type
immobilization condition
ligand density
experimental buffers
experimental temperatures
analyte concentrations
regeneration condition
figure of binding responses with fit
overlay of replicate analyses
model used to fit the data
binding constants with standard errors