Overview of molecular interactions using Biacore

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Abstract. Surface Plasmon Resonance (SPR) technology is a powerful tool for studying a wide range of different putative interactions. This kind of optical biosensors allow to obtain (in real time and without labelling) quantitative and qualitative information about the kinetics of the surface-binding process. The most critical points to keep in mind when using the technique are presented, as well as practical examples of applications.

1. Introduction

Putative molecular interactions demonstrated using different partners and different methods [1] is the best way to elucidate what is going on in a biological system. There are a wide range of methods available for detecting interactions (e.g., ELISA, pull downs, radio and fluorescence-ligand assays) useful for measuring high-affinity interactions. There are several label-free approaches for the detection of biomolecular interactions based on physical principles such as surface plasmon resonance (SPR), interferometry, diffraction and quartz microbalance. Those measurements are not possible using any other technology [2]. Among these, SPR based technology is the most widely used. In addition, SPR allows the analysis of weak interactions saving time and sample and having high throughput screening.

The analysis of molecular interactions using SPR is becoming the most used technique to study interactions between macromolecules without labeling and in real time. It helps us to answer different questions such as:

- How specific is an interaction?
- How strong is an interaction?
- How fast is an interaction (in terms of association and dissociation rates).
- What are thermodynamic parameters for an interaction?

As well mentioned in [3], when studying molecular interactions, the ligand is bound to a solid support, such as a sensor chip. After exposing the ligand to a potential binder (an analyte), the molecules can interact spontaneously. To be able to repeat the measurement in the same chip with different analyte concentrations, the surface of the chip needs to be regenerated. The regeneration agent should be selected by its ability for removing the analyte without significantly damaging the ligand. In addition, the nature of this regeneration agent gives us information about the kind of interaction.

Since the introduction of the first commercial SPR machine in 1990 by Pharmacia Biosensor AB, 25 suppliers of SPR instruments have appeared. Here we will discuss about the Biacore T100 instrument (General Electrics Healthcare), released in 2005 and recently upgraded to T200.

2. Methodology

2.1. Physical principles: What does Surface Plasmon Resonance (SPR) mean?

Total internal reflection (TIR) occurs when polarized light pass at a critical angle through a glass prism on a sensor chip. When a thin -about 50 nm- metal (usually gold) layer is added, photons become plasmons and a reduction (a dip) in the intensity of reflected light can be detected (Fig. 1a, 1b, 1c).

The angle at which the maximum loss of the reflected light intensity occurs is called resonance angle or SPR angle. The angle at which the minimum intensity is observed will shift from A to B. A change in the refractive index at the surface of the gold layer occurs as a result of binding of molecules to the gold layer side [4]. The refractive index in the side of the gold it is affected by the amount of mass bounded to the ligand. Fig. 2 shows a typical sensorgram:

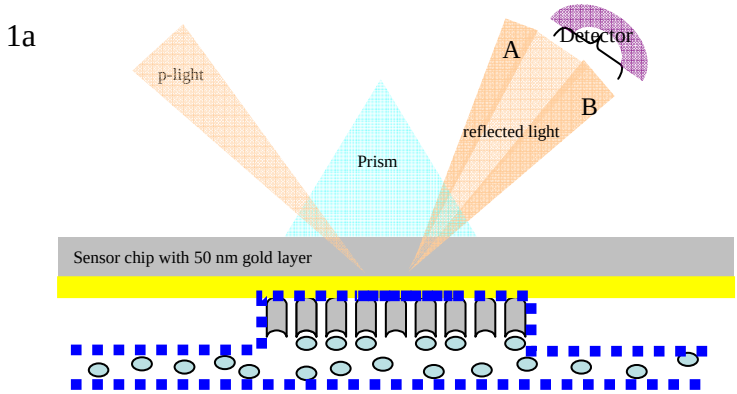

Figure 1a) The polarized light illuminates the sensor chip under conditions of total internal reflection. One binding partner (ligand) is immobilized on the chip, and the analyte is injected. Binding between the partners is increases the mass concentration at the surface of the chip resulting in an increase in the refractive index of the solution close to the surface and a shift in the position of the resonance angle (from A to B).

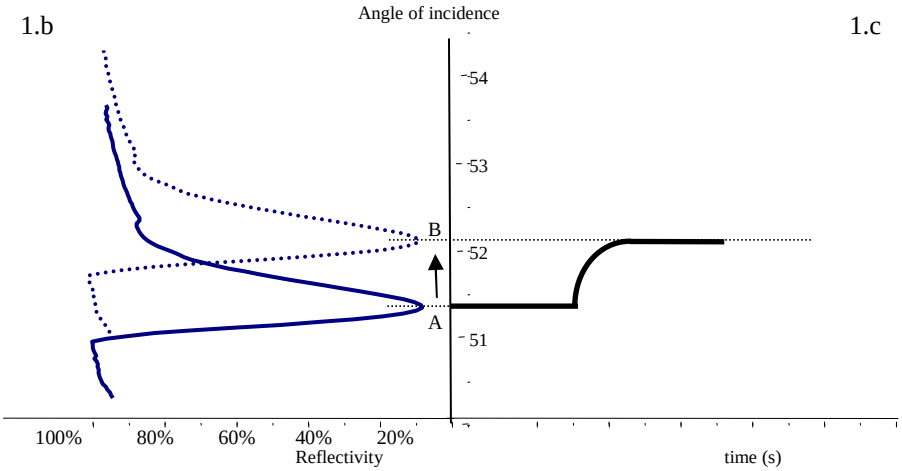

Figure 1b) Rotate SPR dips: reflected light versus incident angle. After the change in refractive index (binding), the angle changes and a shift of the SPR angle to position B appears. **Figure 1c)** Plot of the angle shift as a function of time (sensorgram).

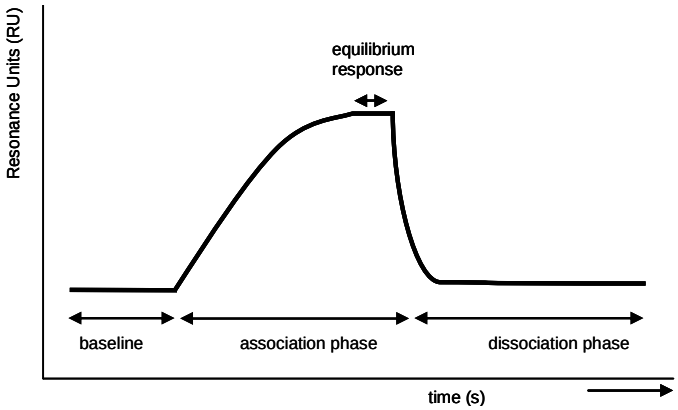

Figure 2) Sensorgram: the kinetics of the interaction can be studied in real time. The response increases during the association phase when the analyte pass across the flow cell and binds to the immobilized ligand. The end of the injection (association phase) might correspond to the equilibrium. After the injection stop (flow and time defined prior on the software) the running buffer is injected and the response decreases during the dissociation phase, where the analyte is spontaneously dissociating from the ligand.

2.2. Terminology

The terminology used in this area in listed in Table 1.

Table 1. Terminology used in Surface Plasmon technology

Resonance Units (RU)	1.000 RU (Resonance Units) correspond to a shift of 0.1º in the resonance angle, or a change in the refractive index of 10^{-3}. For CM5 chips (standard carboxy-methylation level of the dextran used for general purpose), 1.000 RU corresponds to a surface concentration of 1 ng/mm^2 for proteins. 150KDa corresponds to signals greater than 10.000RU (for CM5 chips).
Sensorgram	A plot illustrating the change of the signal on the surface of the sensor over time. The X-axis represents the time, and Y-axis represents resonances units (RU).
Ligand	The molecule immobilized on the surface of the sensor.
Analyte	The molecule in solution that interacts with the immobilized ligand.
Equilibrium dissociation constant (K_D) and affinity constant (K_A)	Constants representing the affinity between two molecules. They are a function of the concentrations of the complex AB ([AB]) and concentration free of A ([A]) and B ([B]) in the equilibrium state of the mixture of the two molecules A, B. The K_D is the reciprocal of the affinity constant K_A, where K_A=[AB]/[A][B]. Typical range of equilibrium association constants (K_A) is 10^5-10^{12} M^{-1}.
Association rate constant (k_a), dissociation rate constant (k_d)	k_a or k_{on} and k_d or k_{off} represent the rate at which the two molecules A and B associate and dissociate. $A+B \underset{k_d}{\overset{k_a}{\rightleftharpoons}} AB$ Association-rate constants, from 10^3 to 10^8 M^{-1} s^{-1}; and dissociation-range constants, from 10^{-6} to 1 s^{-1}.
Running buffer	Buffer used during the assay. The composition should be adapted according to the nature of the interaction.

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2.3. Commercial chips available

Several chips are available depending on the nature of its surface and binding capabilities: CM5 and CM7 are covered with carboxy-methylated dextran for immobilization via -NH_2, -SH, -CHO, -OH, or -COOH groups. They can attach proteins, nucleic acids, carbohydrates or small molecules. CM4 chips are used when the sample has a high positive charge. CM3 and C1 are indicated for very large analytes; C1 (carboxyl groups attached onto the gold) are recommended when the analyte has avidity for the dextran.

Other chips guarantee the orientation of the ligand: the NTA chip is used for immobilization of histidine-tagged molecules; SA or CAP kit for the immobilization of biotinylated peptides, proteins, nucleic acids, or carbohydates. The Sensor chip L1 is used to incorporate a molecule into a lipid bilayer; it is suitable for work with transmembrane proteins. In the HPA chip, liposomes are adsorbed spontaneously to the hydrophobic surface to form a supported lipid monolayer with the hydrophilic heads directed out toward the solution.

Finally, there are "naked" chips made of untreated gold surface (sensor chip AU) for its use with a wide variety of coating techniques. Customized surface chemistries using self-assembled monolayers (SAM) or other modifications can be designed.

2.4. Instrumentation

The T-100 is a fully-automated instrument (Fig. 3): robotic sampler loader, temperature regulation from 4 to 45 ºC (both for compartment containing the sample as well as the chip), buffer degasser and buffer selector (up to four buffers can be used).

Figure 3: The T-100 system

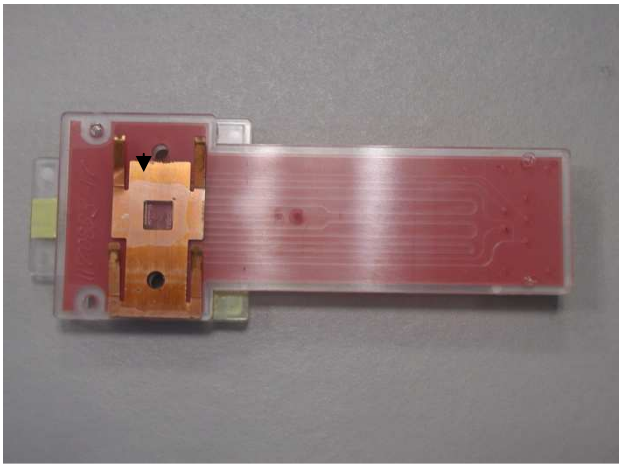

Figure 4: Integrated μ-Fluidic Cartridge (IFC), microfluidifics face.

The main parts of the system are:

- The SPR detection system or refractive index sensor. Biacore uses so-called Kretschmann configuration for the optical detection, with all components fixed. Reflected light intensity is monitored over a range of angles simultaneously; measures are dependent on surface concentrations and temperature.
- The Integrated μ-Fluidic Cartridge (IFC, Fig.4). The IFC is plugged vertically into the frontal part of the machine. The opposite face of the chip is matched with the optointerface (OI) and presented to the prism for optical detection of the signal.The small central square matches the chip and describes four flow cells (Fc). Those flow cells can work in single, pair wise or serial runs. Flow cell 1 can be used as reference cell when it connects to flow cell 2, 3 and 4; whereas Fc3 can be used as a reference for Fc4; these options are selected from the control software.
- Liquid handling system: it consists of two syringes and one peristaltic pump. The buffer selector valve is controlled from the software too (it can be use up to four different buffers); integrated buffer degasser: the running buffer do not need to be degassed prior to use

2.5. Experimental conditions and fitting of mathematical models:

The interpretation of the sensorgrams is not always obvious. The reason why some published data do not fit a simple bimolecular model (A+B=AB) could be the choice of a non recommended experimental design; the shape of the sensorgrams for those simple interactions should be a simple exponential. Square shapes correspond to very fast k_{on} and k_{off}. The deviations from these usual simple exponential shapes are usually caused by instruments artifacts, sample aggregation or other artifact not fully understood. In order to avoid pitfalls hindering these analysis data, it is imperative to be careful with a number of critical aspects: heterogeneity of the sample, mass transport, avidity,

non specific binding, mismatching between buffer running and sample or matrix effects. High flow rates or low density ligand immobilization contribute to minimize mass transport [5].

It is a common mistake to assume the purity of the ligand and analyte. Both of them must be monomeric in solution and form a 1:1 complex when mixed [6]. Basic proteins or sticky samples tend to bind to the dextran matrix; to avoid this problem you can couple the sample onto the surface or use CM4, CM3 or C1 sensor chips (which have a lower charge density) or add carboxymethyl-dextran to the running buffer and samples.

Another important point to consider is coupling, direct or indirect. Direct covalent coupling stabilizes the surface but usually the ligand is randomly oriented throught primaries amines (the heterogeneity onto the surface is raising). Capturing methods using antibodies (anti- GST, anti-Flag) contribute to create a homogeneous surface, but if the capturing step is not stable it may introduce a background surface decay which interferes with the analysis. Selecting the optimum immobilization method is a compromise between introducing surface heterogeneity or instability. It is recommended to use the surfaces at low density ligand, immobilizing an amount of ligand that gives Rmax in the range 20-500 RU (5-20 RU for low molecular weight compounds).

The binding capacity of the surface is a function of different parameters, where:

$$\mathbf{R_L = MW_L/MW_A \times R_{max}/s} \qquad \mathbf{R_{max} = MW_A/MW_L \times R_L \times s}$$

- R_{max} = Maximum binding capacity (in RU). Intensity of the response produced when the ligand is saturated.
- R_L = Response level (RU) of immobilized ligand.
- MW_A = Molecular weight of analyte
- MW_L = Molecular weight of ligand
- s = number of binding sites per ligand

The choice of a good reference surface [7] might correct the bulk refractive index changes, matrix effects, non specific binding, injection noise and baseline drift.

3. Applications

Cutting edge applications are summarized in [8]. There are very different fields where SPR biosensors can be used: drug discovery [9-11], characterization of nucleic acids [12, 13], proteins [14, 15], binding of ligand and receptor [16], determination of the domain of interaction between proteins using mutated proteins or peptides [17]. Biodetection in medicine [18], food and environment [19], development of biosensors of marine toxins using aptamers as a ligands or viral biosensors to detect human pathogens [20] haave been described. Development of vaccines [21], isotyping the immune response [22], or development of biosensor using olfactory receptors expressed in yeast (BOND Project) can also be found.

In the following section some examples of the use of the Biacore T100 in our institution are described:

3.1. Evaluation of dissociation rates after the injection of different peptides through liposomes

L1 chip (dextran with lipophilic substances alkyl chains) [23] was used for this purpose. The scanning electron microscopy (SEM) image of the chip after capturing liposomes is shown in fig.5.

3.2. Kinetics and affinity analysis of small molecule versus enzyme

A drug study on small molecules is presented using a well-known enzyme / inhibitor sytem.

The Biacore T-100 is one of the most sensitive SPR instruments available and can easily detect the binding of small molecules. The analysis can be performed on colored samples (e.g. furosemide) with no interference from absorption or scattering. In addition, when we use high-flow rate (>30 µl/min), we can achieve in less than 1 s the 100% of the sample concentration in the flow system. In this case, the detection and measurement was done in a short time. The methods used are summarized below in Table 2.

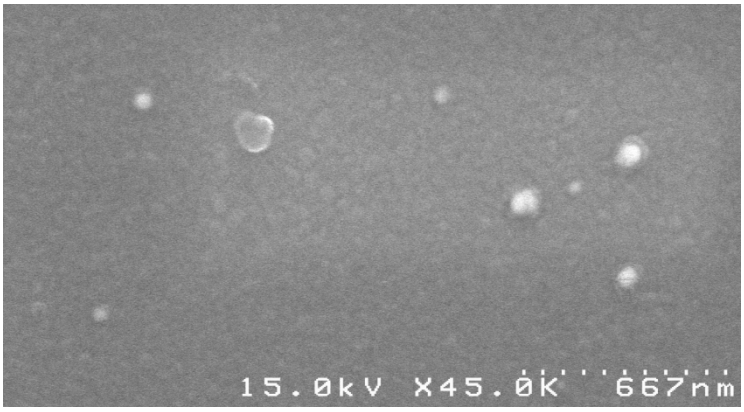

Figure 5. SEM micrograph of liposomes captured on the surface of a L1 chip. Liposomes (100nm) remain intact and circular after being attached to the surface.

Table 2. Methods used in a drug study

	Name	Molecular Weight	Reference
Ligand	human carbonic anhydrase II	30.000 Da	Sigma C# 2522
Analyte	Furosemide	331 Da	Sigma F# 4831
Buffer running	Phosphate buffer saline (PBS) from SIGMA # P4417 + 3% dimethyl sulfoxide (DMSO)		

3.2.1 Immobilization of CAII

The method used for the immobilization of CAII is shown in Table 3. In figure 6, the sensorgrams of immobilization are shown.

Table 3. Method used for the immobilization of CAII

AMINE COUPLING VIA REACTIVE ESTERS	
Before covalent bound: CM5 chip preconditioning: make contact with different pH range and kind of solutions (100mM HCl, 50mM NaOH, 0.1% SDS, water) one injection of each at 100 µl/min during 10s. Electrostatic preconcentration: The pH of the immobilization buffer (very low ionic strength) should be at least 0.5-1 < Isoelectrical point of the ligand to ensure a positive net charge. Elute the electrostatically bounded molecules from the chip surface with an injection of 50mM NaOH, 1M NaCl.	
Amine coupling via reactive esters: Carboiimides have been used to mediate the formation of amine bonds between a carboxylate group (present in the chip) and a primary or secondary amine (present in the ligand).	
EDC	0.4M 1-ethyl-3(3dimethylaminopropyl)-carboiimide in water
NHS	0.1M N-hydroxysuccinimide in water
Make up 1:1 (0.4M EDC/ 0.1M NHS for activating the chip surface) just before coupling. We have to reach 100-150 RU (for CM5 chips) when we have injected the mix during 7 min at 10 µl/min.	
Ethanolamine (deactivate excess reactive groups)	1M ethanolamine-HCl pH8.5. Inject the same time and flow such as an EDC/NHS mixture.
Ligand	20-100 µg/ml in immobilization buffer

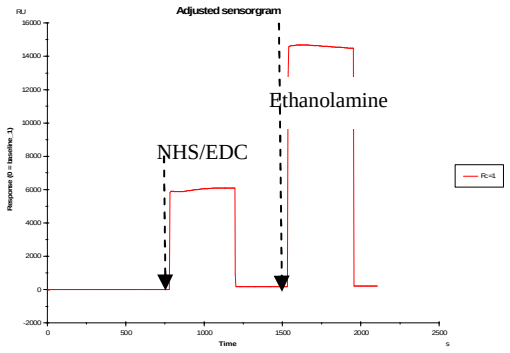

Figure 6a) Reference flow cell after injection of NHS/EDC and ethanolamine (for more detail see amine coupling via amine reactive esters).

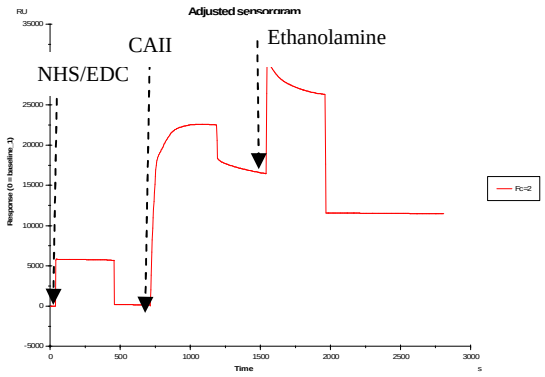

Figure 6b) Immobilization of CAII at 0.1mg/ml diluted in 10mM Sodium Acetat pH 4.9 during 15min at 5µl/min. The relative immobilization level of CAII is 11377 RU.

3.2.2 Steady-State Affinity and Kinetics analysis

As indicated in the formula described in section 2.5, the maximum binding capacity (Rmax) of this model should be: 331/30000*11377*1= 125 RU. However the observed Rmax was 15.4 RU (see Fig. 7). This can be interpreted as only 12% of the CAII being active; higher concentrations of CAII to reach the saturation of the ligand should be used.

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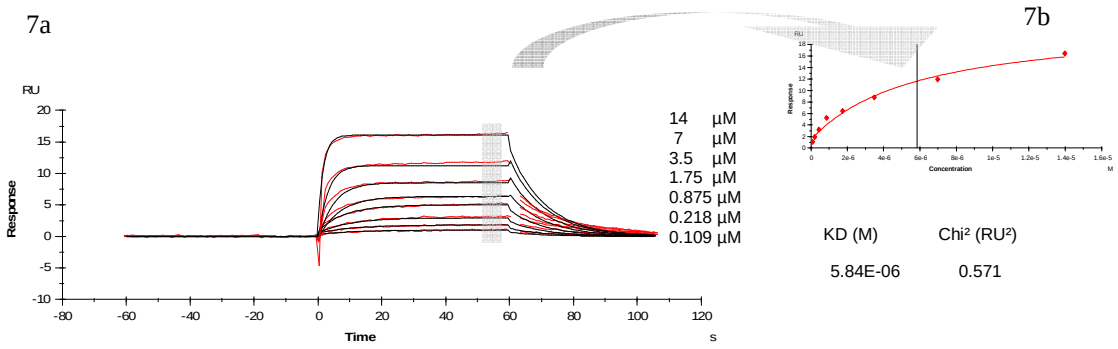
The matching results obtained by kinetics analysis and affinity analysis (Fig. 7a) must be considered. In affinity analysis, is required to reach the steady state (plateau) before the end of the analyte injection almost in one of the injections. It can be achieved by increasing the concentration of analyte injected or elongating the injection time. The affinity plot (Fig. 7b) is a simple isotherm (Y axis: Rmax, X axis: concentration), and K_D is calculated as half of the maximal response. In this example, the instrument, chip, buffer, enzyme immobilization and analyte were prepared as described in [24] using standard amine-coupling chemistry and blocked flow cell surface as a reference (see Fig 6a). Double referencing [6] was applied and the data evaluated using *BiaEval v 1.1 software*.



ka (1/Ms)	kd (1/s)	KD (M)	Rmax (RU)	Flow (µl/min)	Chi² (RU²)
3.54E+04	0.091	2.42E-06	15.4	50	0.23

Figure 7. Kinetics (a) and affinity (b) analysis using (CAII) as a ligand versus its inhibitor furosemide at 25ºC. Data sets were fit to a 1:1 model (black lines) by BiaEval 1.1.

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