Affinity constants from SPR equilibrium analysis

Two ways to assay affinity constants for biomolecular interactions are described. Firstly, the affinity for an immobilized ligand (K_C), and secondly, affinity of the interaction in solution (K_S), using SPR competition experiments. Although affinity constants can be derived from kinetic information, here the equilibrium signal is used, to avoid complications of mass transport limitation and the use of binding models that may not be appropriate for the system under investigation.[1] The cuvette design of the Autolab SPR is pre-eminently suitable to obtain the signal at equilibrium.
The species (a protein or other macromolecule) that is added in solution and binds to the immobilized ligand is defined as the analyte.

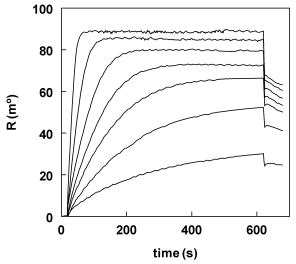
Affinity of an analyte to an immobilized ligand on the SPR sensor chip.

After initialization of the SPR chip, the initial step is coupling of one of the reactants to the SPR chip (see Application Note 31). The conditions can vary much and depend amongst others on isoelectric point and the type of sensor chip. The following is based on EDC/NHS as the coupling strategy. To allow complete access to the analyte-binding site, for small ligands insertion of a spacer between the binding epitope and the sensor dextran matrix is advisable. When a stable surface is needed covalent coupling is preferred over non-covalent immobilization like biotin/avidin coupling. Following the standard EDC/NHS procedure, as a rule of thumb one could start with 1-2 mM ligand reacting during 5-10 min with the activated sensor surface (see Application note 31).

To test the binding capacity of the surface the analyte in a concentration of approximately 10 times the estimated binding constant is injected and the SPR signal at equilibrium assayed. Preferably this would be in the range of 60 to 100 mº. If it is too low the assay will be less accurate. If it is too high, the interaction can be slow, especially with low analyte concentration; the correction for analyte depletion (see below) can be large; and the effects of mass transport will be more evident.

In the actual experiment solutions of analyte are made in a concentration range around the estimated K_D value. Solutions of analyte are made in buffer e.g. HBS buffer pH 7.4. The highest concentration should be approximately 5-10 times the estimated K_D value, the lowest concentration at least equal to K_D, but preferably lower. However, at low concentrations it can take a long time before equilibrium is reached! For an interaction plot at least 5 data-points are needed. Sensorgrams of the analyte interactions are recorded (Fig. 1). From the sensorgrams the SPR signal at equilibrium is determined. This is easily done with the Autolab kinetic software. Make an overlay of the blank corrected curves of interest, position the baseline at the start on zero for all curves, and fit a part of the association phase including a part of the equilibrium signal, with the monophasic association model. The equilibrium signal (R_{eq}) is given by the fit parameters E+R(0). If equilibrium is not reached, e.g. for a low concentration (see Fig. 1), using this method also R_{eq} can be determined. It is always important to check the quality of the fits!

Fig. 1 Binding of Syk tandem SH2 to ITAM



R_{eq} as function of the free analyte concentration can be plotted (Fig. 2) and non-linearly fitted to a one-site binding isotherm (Eq. 1).

$$R_{eq} = \left(\frac{[analyte]_{free}}{[analyte]_{free} + K_C} \right) . R_{\max} \quad (1)$$

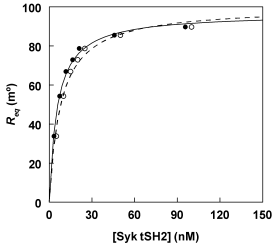
With non-linear fitting programs (not our KE program) readily available now, a non-linear fit is preferred over a linear fit e.g. from a Scatchard plot. The fit yields values for K_C and R_{max}, the value of R_{eq} at saturation of binding.

Depletion correction
In the cuvette-based ESPRIT instrument the concentration of free analyte decreases due to binding to the surface, for this a correction can be made according to Eq. 2.[2]

$$[analyte]_{free} = [analyte]_{total} - \frac{R_{eq}.S.10^{-9}}{122.MW_{analyte}.V_{bulk}} \quad (2)$$

S of the standard sample cell is 2.6 mm^2, V_{bulk} under standard conditions is 35 µL. MW is the molecular weight of the analyte. The effect of depletion correction is shown in Fig. 2: without correction (dotted line, open symbols) K_C in this example is found to be 8.0 nM, and with correction (closed symbols, solid line) 5.5 nM. The depletion

Fig. 2 Binding isotherm with effect of depletion correction



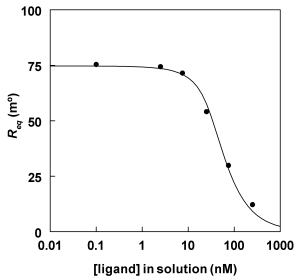
correction can be readily calculated in a spread sheet.

Affinity of an analyte to a ligand in solution.

From SPR competition experiments the equilibrium constant of a molecular interaction in solution (K_S) can be derived. K_S is defined by Eq. 3.

$$K_S = \frac{[analyte]_{free}.[ligand]_{free,solution}}{[analyte-ligand]_{complex,solution}} \quad (3)$$

Fig. 3 Competition assay for solution affinity
25 nM Syk tSH2 binding to ITAM in solution



The experiments are performed with samples containing a constant concentration of analyte in the presence of a concentration range of ligand. R_{eq} determined from the sensorgrams (see section A of this Note) is plotted as a function of the ligand concentration in solution (Fig. 3). In the presence of competing ligand R_{eq} is determined by the affinity to the immobilized ligand and the amount and affinity of the ligand in solution. The amount of sensor-bound analyte is again described by Eq. 1, but now the amount of free analyte is diminished by binding to the ligand in solution. Based on this a modification of Eq. 1 can be derived which fits the data of Fig. 3 [3]. Here the expression of $[analyte]_{free}$ is given for the competition conditions (Eq. 4).

(4)

$$[analyte]_{free} = [analyte]_{total} - \frac{A - \sqrt{A^2 - 4[analyte]_{total}.[ligand]_{total}}}{2}$$

in which $A = [K_S] + [analyte]_{total} + [ligand]_{total}$

Substitution of Eq. 4 into Eq. 1 gives the complete expression, which can be introduced into a non-linear fitting program. It should be noted that this expression contains K_C, therefore the affinity to the sensor surface must be determined (section A of this Note). In the fit the numerical values of $[analyte]_{total}$ and K_C is introduced, and the fit yields values for K_S and R_{max}.

A few remarks concerning the application of this approach:

If binding constants are not needed, and only the relative affinity of ligands has to be established, from the inhibition curves IC_{50}-values can be derived without fitting to the K_S model.

The fixed analyte concentration in the competition experiments is best chosen as 1 to 4 times K_C.

In the fit all concentrations should be in the same unit (M, µM or nM). K_S will be returned in that same concentration unit.

The returned value of R_{max} is the value at complete saturation of all binding sites and should be compatible with the value from the K_C experiment.

Also in these experiments loading of the chip should not be too high, as correction for depletion is not feasible here. For lower MW analytes one has to note especially this point (compare Eq. 2)

In general for a bimolecular interaction the values for K_C and K_S are found to be similar. Deviations can be found for dimer interactions (e.g. GST-fusion proteins), high MW analytes (MW >100 KDa) due to lower partition in the dextran layer of the sensor, and extremely flexible proteins if their conformational freedom is limited by binding to the sensor surface. Deviations between K_S and K_C are also observed if not the proper value of the analyte concentration is used.

The big advantage of this approach is that series of ligands can be investigated using the same sensor surface, without preparing individual surfaces for every ligand.

References

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(2) de Mol, N. J.; Plomp, E.; Fischer, M. J. E.; Ruijtenbeek, R. Kinetic analysis of the mass transport limited interaction between the tyrosine kinase lck SH2 domain and a phosphorylated peptide studied by a new cuvette-based surface plasmon resonance instrument. *Anal Biochem* **2000**, *279*, 61-70.

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