

**Case study**

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**Analytical discrimination of ligands  
in heterogeneous mixtures with  
AFFINImeter**

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**AFFINIMETER**

# Analytical discrimination of ligands in heterogeneous mixtures with AFFINImeter

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*It is not uncommon that the concentration of one or more of the compounds involved in a binding study is not accurately determined. Furthermore, researchers dealing with a mixture of isomers that bind to the same target often assume that there is one single compound in the sample. Under those conditions, fitting ITC isotherms to a 1:1 binding model leads to a completely wrong interpretation of the data. AFFINImeter can be used as an efficient analytical tool to globally analyze sets of ITC isotherms of two competitive ligands that bind the same target, even if their concentration is unknown.*

## Introduction

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Isothermal Titration Calorimetry (ITC) is one of the most commonly used techniques to characterize the binding affinity between molecules. It is the preferred technique in many biophysics and biochemistry labs because it provides a complete thermodynamic characterization of the interactions within a short measurement time and with a moderate amount of sample. ITC can be considered as a *universal detector* because almost every chemical reaction or physical process implies some exchange of heat (or enthalpy)<sup>1</sup>. Taking into account the high sensitivity of modern ITC instruments (able to detect heat exchanges as low as 0.1  $\mu\text{W}$ ); it is not surprising that ITC is used in the study of numerous physicochemical and biological systems: proteins, lipids, surfactants, oligosaccharides, nucleic acids, drugs, etc.

A typical ITC experiment implies a number of successive injections of a ligand solution, from an automated high precision syringe, into a sample cell containing a solution of the receptor molecule. The corresponding isotherm represents the (molar) heat exchanged during each injection as a function of the molar ratio between the ligand and the receptor.

In the simplest case, where one ligand binds to a receptor with one binding site the isotherm has a sigmoidal shape and it should be fitted to a 1:1 binding model to extract the association constant ( $K_A$ ), the binding enthalpy ( $\Delta H$ ) and a parameter ( $r$ ) that coincides with the stoichiometry of the complex only when both ligand and receptor are 100% active and their nominal concentration is accurately determined:

$$r = n \cdot \frac{AF_{receptor}}{AF_{ligand}}$$

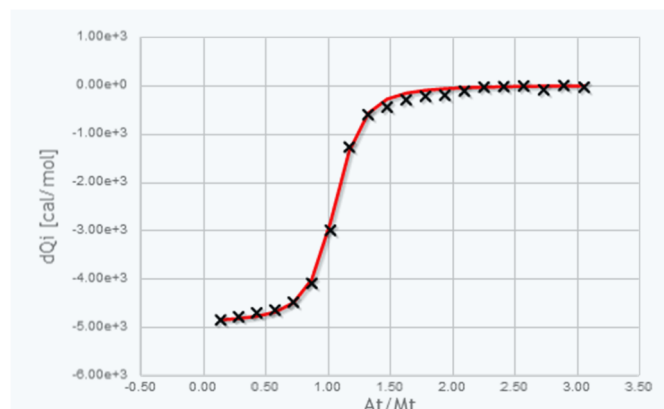
where  $n$  is the stoichiometry and  $AF=[active]/[total]$ . The equivalence between “ $r$ ” and the stoichiometry is not valid, for instance, in cases where a significant percentage of the receptor cannot bind the ligand due to a (partial) unfolding, an irreversible conformational change, or to a chemical degradation of the molecule.

AFFINImeter introduces additional scaling parameters that may also be fitted to correct for the concentration of the receptor in the sample cell ( $r_M$ ) or the concentration of the ligand in the syringe ( $r_A$ ) when any of these concentrations has not been previously determined with enough accuracy.

ITC is extremely sensitive to the concentration of the molecules involved in the experiment. A serious problem that should be faced by ITC users when characterizing binding interactions arises when the ligand solution is not pure and/or the exact concentration in the sample is unknown. Moreover, ligands are often synthesized as a mixture of isomers that may bind the same receptor albeit with different affinity and/or heat exchange. This scenario was pointed out by T. Lundbäck et al.<sup>2</sup> as one of the main problems in hit-to-lead processes of drug discovery programs in pharmaceutical industries.

Normally, the ITC isotherm obtained from the interaction of a mixture of two or more ligands that compete to bind the same receptor exhibits a non-sigmoidal shape that cannot be fitted with the simple 1:1 binding model. The situation becomes more problematic when both ligands interact with the receptor with similar thermodynamic parameters (i.e. two isomers where  $K_1 \approx K_2$  or  $\Delta H_1 \approx \Delta H_2$ ). In such cases the isotherm might show a sigmoidal shape, hiding the competing interactions and, therefore, the presence of more than just one ligand in the sample. Particularly, when the binding enthalpies are similar and the binding constants are different, the isotherm usually

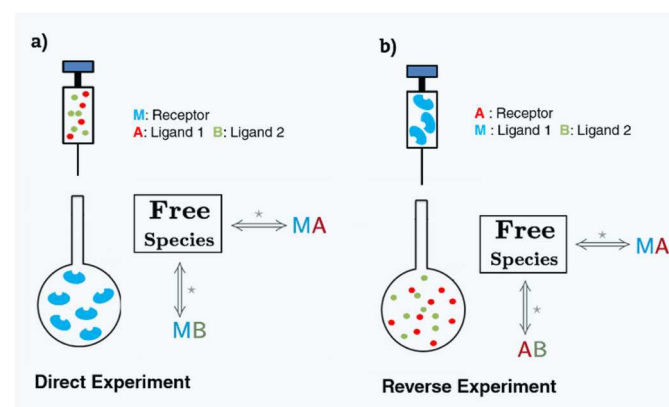
can be fitted with 1:1 binding model and the calculated  $K_A$  will be close to the real  $K_A$  value of the weaker ligand; leading the researcher to a completely wrong conclusion (see Fig. 1 and Table I).



**Fig. 1.** ITC isotherm resulting from the titration of an equimolar mixture of two ligands (A and B) with total concentration  $[A] + [B] = 24$  mM into a receptor solution (M) with  $[M] = 1.2$  mM when both ligands interact with a very similar  $\Delta H$  and different  $K_A$ . The red line shows the best fitting to a 1:1 model (see Table I).

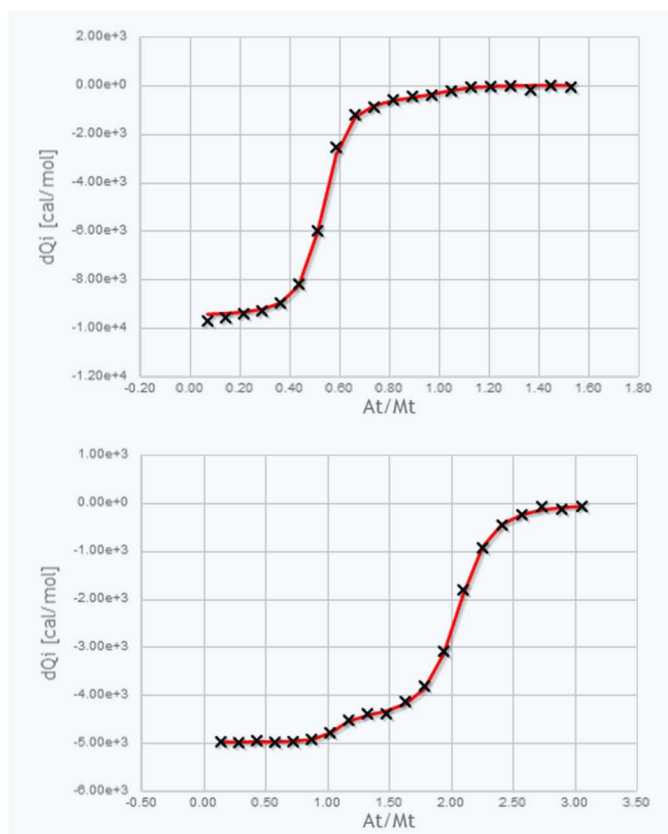
### Two competitive ligands with similar binding enthalpy and different association constant interacting with the same receptor

To overcome with this situation, it is recommended to perform an additional experiment consisting on a reverse titration where the mixture of ligands is placed in the sample cell and the receptor is titrated from the syringe (Fig. 2).



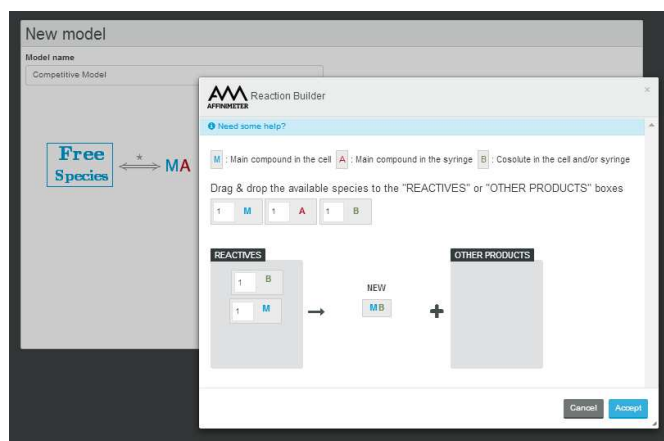
**Fig. 2.** Graphical representation of the (a) direct and (b) reverse competitive experiments described in the text, together with the corresponding binding models. \*

If the final molar concentration of the receptor in the cell is in excess, the isotherm should be a two-stepped curve instead of a standard sigmoidal shape. This reveals the presence of the two binding events (Fig 3).



**Fig.3.** Simulated ITC isotherms of the direct competitive (a) and reverse (b) experiments, and global fitting (red curve) with AFFINImeter to the binding models of Fig.2.

AFFINImeter offers an advanced fitting tool that can be used to globally fit the data of direct and reverse competitive experiments using models that can be easily designed by the user with the “Model Builder” tool. This easy-to-use interface allows writing a set of coupled equilibrium reactions that describes the binding processes taking place during each experiment (Fig.4). In this way, the reaction schemes in figure 2 were created to describe both direct and reverse competitive experiments.



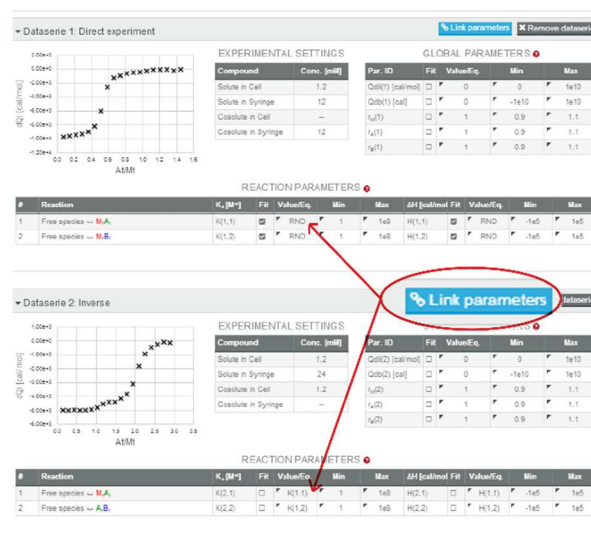
**Fig. 4.** Screenshot of the “Model Builder” interface of AFFINImeter.

The equilibrium constants and enthalpies of identical reactions in different curves must be simultaneously fitted to the same values. Thus, in order to do a proper analysis without overparameterizing the model, the following restrictions were applied in the “Fit Settings” form of AFFINImeter (note that the “Link” button may be employed to facilitate this, Fig.5):

$$K(MA)_{\text{direct}} = K(MA)_{\text{reverse}}$$

$$K(MB)_{\text{direct}} = K(AB)_{\text{reverse}}$$

$$\text{and } \Delta H(MA)_{\text{direct}} = \Delta H(MA)_{\text{reverse}}$$



**Fig. 5.** Screenshot of the “Fit Setting” interface showing the “Link” button to relate thermodynamic parameter of different isotherms

By using this model it is possible to simultaneously fit both isotherms by using 4 parameters (Figs 4 and 5, and Table I).

**Table I. Thermodynamic data of the analysis of isotherm in Fig. 2 and 3 with different binding models.**

|                   | $K_1(\text{M}^{-1})$                | $\Delta H_1$ (Kcal/mol) | $K_2(\text{M}^{-1})$              | $\Delta H_2$ (Kcal/mol) |
|-------------------|-------------------------------------|-------------------------|-----------------------------------|-------------------------|
| 1:1 Model         | $7.3 \cdot 10^4 \pm 3 \cdot 10^3$   | $-4.907 \pm 9$          | -                                 | -                       |
| Competitive Model | $5.369 \cdot 10^4 \pm 7 \cdot 10^2$ | $-4.524 \pm 5$          | $1.2 \cdot 10^7 \pm 1 \cdot 10^6$ | $-4.974 \pm 3$          |

## Two competitive isomers of unknown relative concentration interacting with the same receptor

AFFINImeter provides the analytical tools to evaluate ITC isotherms of a mixture of two isomers binding to a receptor even if the molar ratio of the ligands in the mixture is unknown. In fact, this is a situation that researchers might encounter frequently: the total concentration of the mixture ([L]) is known while the exact concentration of the ligands ([A] and [B]) is uncertain.

AFFINImeter allows the independent fitting of scaling factors for: (i) the concentration of the main solute in the sample cell ( $r_M$ ); (ii) the main solute in the syringe ( $r_A$ ); and (iii) the co-solute that can be in the cell or/and in the syringe ( $r_B$ ). For the present case, assuming that the receptor has only one binding site, that all the species are 100 % active and that the nominal concentration of the ligands are equal to the total concentration of the mixture (which is rigorously valid only in the case of a mixture of compounds with identical molecular mass such as isomers),  $r_A$  and  $r_B$  account for the correction in the concentration of each ligand respect to the total concentration [L], i.e.  $[A] = r_A \cdot [L]$  and  $[B] = r_B \cdot [L]$ .

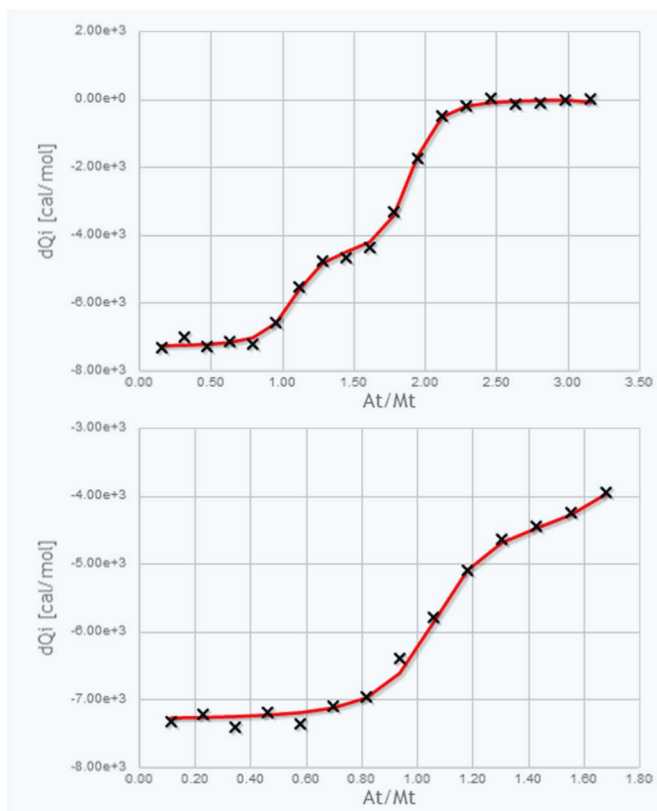
AFFINImeter also allows establishing dynamic mathematical relations within fitting parameters. In this case it is convenient to apply the following restriction:

$$r_B = 1 - r_A$$

to ensure that the sum of the ligand concentrations equals the total molar concentration of the mixture.

The risk of overparametrization increases with the number of the parameters required for the analysis. Thus, performing a global fitting of several isotherms registered under different experimental conditions is strongly recommended to obtain an accurate and reliable result.

In order to illustrate the process described in this section, two ITC isotherms were obtained: (i) the titration of a mixture of two isomers with a total concentration of 22 mM into a 1 mM solution of a receptor; and (ii) the titration of the same mixture at 14.7 mM into a 1.1 mM solution of the same receptor (Fig 6).



**Fig. 7.** Simulated ITC isotherms (cross marks) and global fitting (red line) of the titration of a mixture of ligands where (a) the total ligand concentration [L] = 22 mM and the receptor concentration [M] = 1 mM; (b) [L] = 14.6-mM into a receptor solution at [M] = 1.1 mM.

Both isotherms were analyzed globally with the competitive binding model shown in Fig. 2a. In order to remove unnecessary degrees of freedom, the following dynamic restrictions were applied (in the “Fit Settings” form of AFFINImeter):

$$K(\text{MA})_1 = K(\text{MA})_2$$

$$K(\text{MB})_1 = K(\text{MB})_2$$

$$r(\text{A})_1 = r(\text{A})_2 = 1 - r(\text{B})_1 = 1 - r(\text{B})_2$$

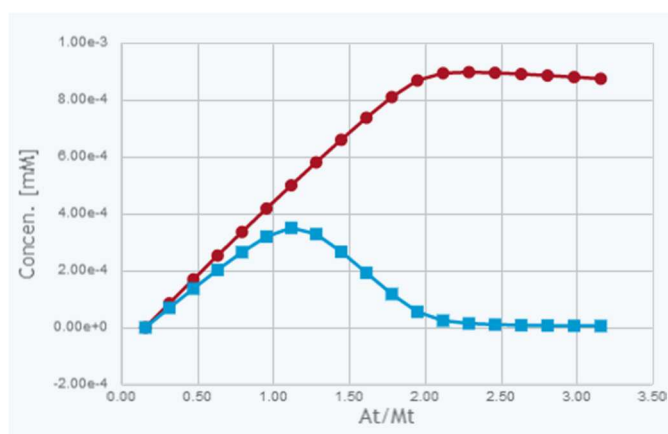
$$\Delta H(\text{MA})_1 = \Delta H(\text{MA})_2$$

$$\Delta H(\text{MB})_1 = \Delta H(\text{MB})_2$$

The nominal concentration of each isomer in the syringe was set to the known value of the total mixture concentration [L] to allow the fitting algorithm freely searching for the right concentrations throughout the scaling parameters.

The red lines in Fig. 7 show the best fitting to the ITC isotherms, and Table II includes the calculated parameters. The results indicate the presence of two ligands mixed at a molar ratio 1:1.2. The ligand present in excess (54%) has high affinity for the target ( $K=1.7 \cdot 10^7 \text{ M}^{-1}$ ) and interacts with a high enthalpy ( $\Delta H = -11.1 \text{ kcal/mol}$ ). The second ligand interacts weakly with the target ( $K = 4.9 \cdot 10^4 \text{ M}^{-1}$ ) and with a

moderate binding enthalpy ( $\Delta H = -2.9 \text{ kcal/mol}$ ). For a better interpretation of the ITC results, AFFINImeter also provides the distribution of all the complex species involved in the interactions as a function of the concentration ratio in the cell between A and M. The blue line in Fig. 9 represents the concentration-of the complex formed between the receptor and the low affinity isomer while the red line represents the concentration of the complex involving the high affinity isomer. At the beginning of the titration, both isomers bind the receptor. When the receptor becomes saturated ( $A_t/M_t \sim 1$ ) the high-affinity isomer displaces the weak-affinity isomer.



**Fig.9.**Species distribution of the complex formed of the receptor with the low affinity isomer (blue) and with the high-affinity isomer (red).

**Table II. Fitting parameters of the global analysis of the isotherms shown in Figs 5 and 6.**

|                   | $r_1$ | $K_1 (\text{M}^{-1})$             | $\Delta H_1 (\text{kcal/mol})$ | $r_2$ | $K_2 (\text{M}^{-1})$             | $\Delta H_2 (\text{kcal/mol})$ |
|-------------------|-------|-----------------------------------|--------------------------------|-------|-----------------------------------|--------------------------------|
| Competitive Model | 0.546 | $1.7 \cdot 10^7 \pm 1 \cdot 10^6$ | $-11.114 \pm 0.015$            | 0.453 | $4.9 \cdot 10^4 \pm 3 \cdot 10^3$ | $-2.841 \pm 0.026$             |

## Conclusions

AFFINImeter offers new functionalities that allow the analysis of complex ITC isotherms, such as those describing the interaction of a mixture of competing ligands binding to the same receptor.

To illustrate this, two examples have been shown: (i) a global analysis of two isotherms obtained with a direct and a reverse ITC experiments where two competing ligands that bind to the same receptor with similar

binding enthalpy and different affinity; and (ii) a global analysis of two ITC isotherms registered when a mixture of two isomers of unknown molar ratio compete for the same target.

AFFINImeter can be used as an efficient analytical tool to determine the molar ratio of the isomers present in the mixture.

## Thermodynamic parameters and experimental conditions used to simulate isotherms

Fig. 1 and 3

Cell Volume = 1.403 mL

Injection volume = 10 nj

$C_M=1.2$  mM,  $C_A=12$  mM and  $C_B=12$  mM

$K(MA)=5 \cdot 10^4$  M<sup>-1</sup>  $\Delta H(MA)=-4500$  cal/mol

$K(MB)=1.2 \cdot 10^7$  M<sup>-1</sup>  $\Delta H(MB)=-5000$  cal/mol

Fig. 4

Cell Volume = 1.403 mL

Injection volume = 10 nj

$C_M=1.2$  mM,  $C_A=24$  mM and  $C_B=1.2$  mM

$K(MA)=5 \cdot 10^4$  M<sup>-1</sup>  $\Delta H(MA)=-4500$  cal/mol

$K(AB)=1.2 \cdot 10^7$  M<sup>-1</sup>  $\Delta H(AB)=-5000$  cal/mol

Fig. 5

Cell Volume = 1.400 mL

Injection volume = 12 nj

$C_M=1$  mM,  $C_A=12$  mM and  $C_B=10$  mM

$K(MA)=20 \cdot 10^6$  M<sup>-1</sup>  $\Delta H(MA)=-60000$  cal/mol

$K(MB)=60 \cdot 10^3$  M<sup>-1</sup>  $\Delta H(MB)=-1500$  cal/mol

Fig. 6

Cell Volume = 1.400 mL

Injection volume = 12 nj

$C_M=1.1$  mM,  $C_A=8$  mM and  $C_B=6.6$  mM

$K(MA)=20 \cdot 10^6$  M<sup>-1</sup>  $\Delta H(MA)=-60000$  cal/mol

$K(MB)=60 \cdot 10^3$  M<sup>-1</sup>  $\Delta H(MB)=-1500$  cal/mol

A slight noise was added to the simulated curves. The molar enthalpies per titration of the input file to fit the data of Fig. 5 and 6 were modified to simulate a data obtained when only the total concentration of the titrated ligands was known.

## References

<sup>1</sup> M.W. Freyer and E.A.Lewis, Isothermal Titration Calorimetry: Experimental design, data analysis and probing macromolecule/ligand binding and kinetic interactions. Methods in cell Biology. **2008**, Vol.84 pp 79-112

<sup>2</sup> T. Lundbäck, R. O´Brien and G. Williams. In the Mix: Simultaneous Affinity Determination for Isomers and Enantiomers. Microcal Application Note. 2006, pp 1-12

\* Note that the label of the different compounds are fixed in the current version of AFFINImeter: M is the main compound in the sample cell, A is the main compound in the syringe and B is the co-solute in the cell or/and in the syringe.



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