NOTE

Stoichiometric and site constants: two approaches to analyze data with AFFINImeter.

I. The concepts of stoichiometric and site binding constants

The interaction between a monovalent ligand *L* and a multivalent receptor *R* involves the presence of various species, including the complex of *R* fully saturated with a number of ligands, and intermediate complexes of *R* partially saturated. This scenario can be described in terms of reaction schemes following two approaches: a) based on equilibria between existing stoichiometric species and b) based on equilibria between *L* and specific interaction sites of *R*. For a better understanding, let´s consider a particular case where *L* binds to a bivalent receptor:

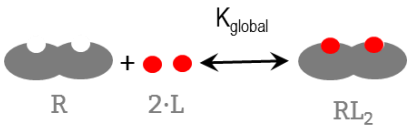
a) Approach based on equilibria between the existing stoichiometric species:

$$K_1 = \frac{[RL]}{[R]\cdot[L]};\ K_2 = \frac{[RL_2]}{[RL]\cdot[L]}$$

Fig.1 Diagram of binding equilibria based on stoichiometric species.

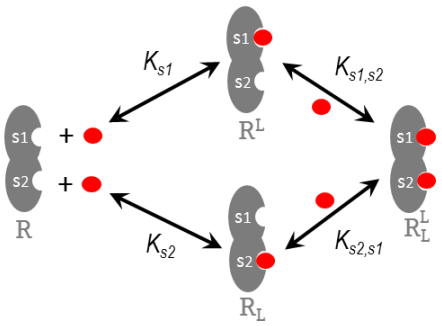
Here, the reaction scheme includes a first equilibrium between the free species and the intermediate *RL* and a second equilibrium between *RL* + *L* and *RL₂* (Fig. 1). The corresponding binding constants, K_1 and K_2, are denominated **stoichiometric binding constants**[1] since they refer to equilibria between stoichiometric species[2].

We could also describe the formation of the bivalent complex, RL_2, in a single step using a global equilibrium constant, K_{global}, which value is the product of the stoichiometric constants:



$$K_{global} = \frac{[RL_2]}{[R]\cdot[L]^2}; \quad K_{global} = K_1 \cdot K_2$$

b) An approach based on equilibria between L and specific interaction sites of R:



$$K_{s1} = \frac{[R^L]}{[R]\cdot[L]}; \; K_{s2} = \frac{[R_L]}{[R]\cdot[L]}$$

$$K_{s1,s2} = \frac{[R_L^L]}{[R^L]\cdot[L]}; \; K_{s2,s1} = \frac{[R_L^L]}{[R_L]\cdot[L]}$$

Fig.2 Diagram of binding equilibria based on ligand – site interactions.

In this case, the reaction scheme considers the presence of two sites in the bivalent receptor and two intermediate complexes (R^L and R_L) formed when the ligand binds to s1 or s2 and consequently, the existence of a total of 4 equilibria (Fig. 2). The corresponding binding constants, K_{s1}, K_{s2}, $K_{s1,s2}$ and $K_{s2,s1}$, are denominated **site binding constants**[3], since they refer to equilibria between *L* an each specific site of *R*.

Note that, stoichiometric and site binding constants describe the same overall binding event, but represent different equilibria. Stoichiometric binding constants do not distinguish between specific binding sites. The intermediate stoichiometric species *RL* (Fig. 1) comprises the contribution of the intermediates R^L and R_L (Fig.2). Hence, the stoichiometric constant K_1 covers the information of the two site constants K_{s1} and K_{s2}. The same reasoning can be applied to K_2 and $K_{s1,s2}$ /$K_{s2,s1}$. Ultimately, the values obtained of stoichiometric constants will be different from the values obtained of site constants, yet they can be correlated based on the law of mass action[4] where $[RL] = [R^L] + [R_L]$. Thus, for the above example **the relation between constants is:**

$$K_1 = K_{s1} + K_{s2};\quad K_2 = \frac{K_{s1,s2} \cdot K_{s2,s1}}{K_{s1,s2} + K_{s2,s1}}$$

II. Stoichiometric and site binding constants approaches applied to independent and non-independent sites

The equilibria shown in Figs. 1 and 2 represent the same binding event where 2 molecules of *L* bind to the bivalent receptor *R*. Now, from these two approaches and based on the values of the binding constants obtained it is possible to determine if there is a dependency between binding sites.

When the interaction of *L* with the bivalent R is described by a site approach, the most general case involves the description of four binding constants, K_{s1}, K_{s2}, $K_{s1,s2}$ and $K_{s2,s1}$ (see Fig. 2). In a particular case of **independent sites**, that is when the interaction of *L* with s1 does not influence the interaction of *L* with s2 and vice versa, K_{s1} equals $K_{s2,s1}$ as they describe the same equilibrium; similarly, K_{s2} equals $K_{s1,s2}$. In this situation the relationship between stoichiometric and site constants turns into:

$$K_1 = K_{s1} + K_{s2} \qquad K_2 = \frac{K_{s1} \cdot K_{s2}}{K_{s1} + K_{s2}}$$

Moreover, **if the sites are equivalent** K_{s1} equals K_{s2}, the overall binding is described by two stoichiometric constants (K_1 and K_2) and a single site constant (K_{s1}) where:

$$K_1 = 2 \cdot K_{s1}\,; \qquad K_2 = \frac{K_{s1}}{2}$$

III. Approaches for binding model design in AFFINImeter: stoichiometric equilibria and independent sites

The design of binding models for ITC curve fitting with AFFINImeter can be performed following two different approaches, based on stoichiometric or site binding constants. This way, a new FIT or SIM subproject has to be designed as “Stoichiometric equilibria” or “Independent sites” to use an approach based on stoichiometric constants or site constants,

respectively. The following paragraph describe the specific characteristics of these two options:

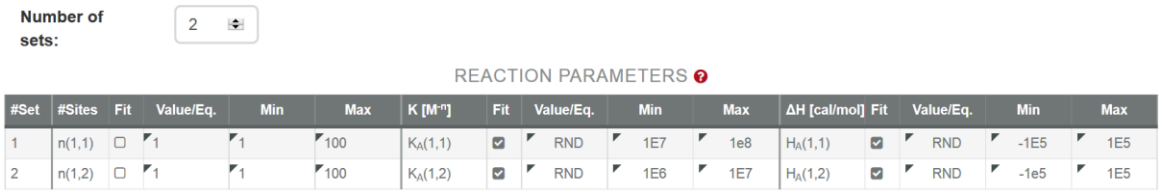
a) STOICHIOMETRIC EQUILIBRIA approach[5]

This approach uses reaction schemes based on equilibria between stoichiometric species and yield stoichiometric binding constants. The reaction schemes are designed with the reaction builder functionality. A model based on stoichiometric equilibria is valid to fit data of both independent and non-independent events and therefore, it is of wider applicability.

Fig.3 Model based on stoichiometric equilibria, describing the reaction scheme of Fig. 1.

b) INDEPENDENT SITES approach

This approach uses a reaction scheme based on the binding of the ligand to individual sites and considering that **all the sites are independent**; thus, it supplies site binding constants. This project type does not use the reaction builder. Instead, it offers a sole reaction scheme where a receptor with a certain number of sites "n" binds to the ligand. The sites are grouped into sets to discern between sites that are non-equivalent. i.e . the interaction of the bivalent *R* interacting with the monovalent *L* would be described with a model of one set of two sites if s1 a s2 are equivalent or, with two sets of one site each if s1 and s2 are different (Fig. 4). Moreover, the number of sites in each set can be considered as a fitting parameter (for cases where "n" is unknown). An AFFINImeter model based on independent sites is NOT valid to fit data when there is a dependency between sites (i.e. cooperative interactions).


Number of sets: 2

REACTION PARAMETERS

#Set	#Sites	Fit	Value/Eq.	Min	Max	K [M^{-n}]	Fit	Value/Eq.	Min	Max	ΔH [cal/mol]	Fit	Value/Eq.	Min	Max
1	n(1,1)	☐	1	1	100	$K_A(1,1)$	☑	RND	1E7	1e8	$H_A(1,1)$	☑	RND	-1E5	1E5
2	n(1,2)	☐	1	1	100	$K_A(1,2)$	☑	RND	1E6	1E7	$H_A(1,2)$	☑	RND	-1e5	1E5

Fig.4 Reaction parameters of a binding model based on an independent sites approach, describing the reaction scheme of Fig. 2 and considering that s1 and s2 are non-equivalent.

Interacting systems with a defined number of independent sites can also be analyzed using a model based on the stoichiometric equilibria approach (Table I). However, the use of the independent sites approach should be the first choice to analyze this type of systems. In one hand, the independent sites approach provides true, specific information of each ligand – site interaction that is missing in the stoichiometric equilibria approach. Additionally, in most of cases it allows to dramatically reduce the number of parameters describing the thermodynamic events experienced by the system during the experiment.

Table I. Characteristics of the two approaches for binding model design available in AFFINImeter.

STOICHIOMETRIC APPROACH	INDEPENDENT SITES APPROACH
• Based on stoichiometric equilibria.	• Based on ligand – site equilibria.
• Provides stoichiometric binding constants.	• Provides site binding constants.
• Uses the reaction builder to design the models.	• Uses a pre-defined model where binding sites are grouped in sets with identical K and ΔH
• The model has to include all the stoichiometric species existing in the binding event.	• Does not considerate intermediate complex species.
• The stoichiometry of each species is a known, constant parameter, defined in the reaction scheme.	• The stoichiometry (number of sites) can be a fitting parameter.
• Valid for fitting: ○ Interacting systems with independent sites of defined stoichiometry. ○ Interacting systems with non-independent sites (i.e. cooperative interactions) of defined stoichiometry. ○ Competitive interactions between two ligands.	• Valid for fitting: ○ Interacting systems with independent sites of defined stoichiometry. ○ Interacting systems with independent sites of undefined stoichiometry. ○ Competitive interactions between two ligands.
• NOT valid for: ○ Interacting systems with independent or non-independent sites of undefined stoichiometry.	• NOT valid for: ○ Interacting systems with non-independent sites (i.e. cooperative interactions).

References and comments

[1] An alternative name of the stoichiometric binding constants is “stepwise binding constants”.

[2] An stoichiometric species is understood as a species with an elemental composition that can be represented by integral numbers.

[3] An alternative name of the site binding constants is “microscopic binding constants”.

[4] **K.A. Connors** “Binding constants: the measurements of molecular complex stability”; Willey interscience; New York, 1987. pp 21-24.

[5] The binding models based on stoichiometric equilibria have been traditionally named as “sequential binding” models in other analysis software such as Microcal Origin.

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