Supporting Information

Differential Binding Models for Isothermal Titration Calorimetry: Moving beyond the Wiseman Isotherm

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A. General Derivation of the Total Differentials *df*/*d*Φ

The overall equilibrium reaction for a homotropic and homonuclear binding system can be written in terms cumulative binding constants (β_i), as given by

$$\mathrm{M} + i\mathrm{X} \rightleftarrows \mathrm{MX}_i \qquad \beta_i = \frac{[\mathrm{MX}_i]}{[\mathrm{M}][\mathrm{X}]^i} \tag{S1}$$

then, the binding polynomial for a receptor with *n* binding sites is given by

$$P_M = 1 + \beta_1[\mathrm{X}] + \beta_2[\mathrm{X}]^2 + \ldots + \beta_n[\mathrm{X}]^n = \sum_{i=0}^{i=n} \beta_i[\mathrm{X}]^i \tag{S2}$$

where by definition $\beta_0 = 1$ (i.e., $\beta_0 = [\mathrm{M}]/[\mathrm{M}]$).

The binding polynomial describes the distribution of the binding species in solution and it can be used to calculate the average number of ligands bound per receptor $\bar{N}_X$ and the binding capacity B_X, as given by following the expressions

$$\bar{N}_X = \left(\frac{\partial \ln P_M}{\partial \ln[\mathrm{X}]}\right)_{T,P} = \frac{\sum_{i=0}^{n} i\beta_i [\mathrm{X}]^i}{P_M} \tag{S3a}$$

$$\begin{aligned} B_X &= \left(\frac{\partial^2 \ln P_M}{\partial(\ln[\mathrm{X}])^2}\right)_{T,P} = \left(\frac{\partial \bar{N}_X}{\partial \ln[\mathrm{X}]}\right)_{T,P} \\ &= \frac{\sum_{i=0}^{n} i^2\beta_i[\mathrm{X}]^i}{P_M} - \left(\frac{\sum_{i=0}^{n} i\beta_i[\mathrm{X}]^i}{P_M}\right)^2 \end{aligned} \tag{S3b}$$

In addition, the binding polynomial can be used to represent the mole fraction (α_i) of a receptor bound to *i* ligand species

$$\alpha_i = \frac{[\mathrm{MX}_i]}{M_T} = \frac{\beta_i[\mathrm{X}]^i}{P_M} \tag{S4}$$

The term α_i corresponds to the normalized probability of finding the complex MX_i in solution. The partial differential of $\ln \alpha_i$ with respect to $\ln[\mathrm{X}]$ for a system at constant temperature and pressure is given by

$$\overline{F}_i \;=\; \left(\frac{\partial \ln \alpha_i}{\partial \ln[\mathrm{X}]}\right)_{T,P} \;=\; i - \overline{N}_X \tag{S5}$$

The term $\overline{F}_i$ describes the rate of change in the chemical potential of a receptor bound to *i* ligands with respect to the change in the change in the chemical potential of the free ligand in solution.

The mass balance equations for a homotropic binding system represent the two independent variables for the titration (i.e., M_T and X_T). Each mass balance equation can be expressed in terms of the binding polynomial (P_M) as

$$\begin{aligned} M_T &= [\mathrm{M}]+[\mathrm{MX}]+[\mathrm{MX}_2]+\ldots+[\mathrm{MX}_n] \\ &= [\mathrm{M}]\sum_{i=0}^{n}\beta_i[\mathrm{X}]^i = [\mathrm{M}]\cdot P_M \end{aligned} \tag{S6}$$

and

$$\begin{aligned} X_T &= [\mathrm{X}]+[\mathrm{MX}]+2\cdot[\mathrm{MX}_2]+\ldots+n\cdot[\mathrm{MX}_n] \\ &= [\mathrm{X}]+[\mathrm{M}]\sum^{n} i\beta_i[\mathrm{X}]^i \end{aligned} \tag{S7a}$$

By substituting the term [M] by M_T / P_M in eq S6, we obtain

$$\begin{aligned} X_T &= [\mathrm{X}]+M_T\frac{\sum^{n} i\beta_i[\mathrm{X}]^i}{P_M} \\ &= [\mathrm{X}]+M_T\cdot\overline{N}_X \end{aligned} \tag{S7b}$$

We can combine the mass-balance expression (eqs S6 and S7) into a single dimensionless expression by dividing eq S7b by the term M_T as given by

$$\Phi \;=\; \frac{[\mathrm{X}]}{M_T}+\overline{N}_X \tag{S8}$$

where Φ corresponds to the degree of titration, as given by the ratio between the terms X_T and M_T (i.e., $\Phi = X_T / M_T$). One important feature of eq S8 is that every term in the sum is dimensionless to facilitate the evaluation of the titration curve from a theoretical perspective.[1,2] Therefore, implicit differentiation of a function of the form $f([\mathrm{X}])$ at constant M_T can be expressed as:

$$\frac{df}{d\Phi} = \frac{df}{d[\mathrm{X}]}\left(\frac{\partial[\mathrm{X}]}{\partial\Phi}\right)_{M_T} \tag{S9}$$

where f([X]) is any of the dependent variables related to the binding species in solution that is a function of the free ligand concentration, such as (i) the receptor-normalized free ligand concentration (i.e., [X]/M_T), (ii) the mole fractions α_i for the receptor species bound to i ligands, or (iii) the average number of ligands bound per receptor $\bar{N}_X$.

The expression for $M_T^{-1}d[\mathrm{X}]/d\Phi$ is derived by implicit partial differentiation of eq S8 with respect to Φ as given by

$$\begin{aligned} \frac{d\Phi}{d\Phi} &= \frac{1}{M_T}\cdot\frac{d[\mathrm{X}]}{d\Phi}+\left(\frac{d\bar{N}_X}{d[\mathrm{X}]}\right)\cdot\frac{d[\mathrm{X}]}{d\Phi} \quad ,or \\ 1 &= \left(1+M_T\frac{B_X}{[\mathrm{X}]}\right)\left(\frac{1}{M_T}\cdot\frac{d[\mathrm{X}]}{d\Phi}\right) \end{aligned} \tag{S10}$$

therefore,

$$\frac{1}{M_T}\cdot\frac{d[\mathrm{X}]}{d\Phi} = \frac{[\mathrm{X}]}{[\mathrm{X}]+M_T B_X} \tag{S11}$$

The derivative $d\bar{N}_X/d[\mathrm{X}]$ in eq S10 was obtained from S3b as follows:

$$B_X = \frac{d\bar{N}_X}{d\ln[\mathrm{X}]} = [\mathrm{X}]\cdot\frac{d\bar{N}_X}{d[\mathrm{X}]} \tag{S12}$$

where the last term was obtained using the definition for $d(\ln x)$ (i.e., $d\ln x = (1/x)\cdot dx$). Then by rearrangement of eq S12, we obtain the expression for the differential $d\bar{N}_X/d[\mathrm{X}]$ in terms of the binding capacity B_X as given by

$$\frac{d\bar{N}_X}{d[\mathrm{X}]} = \frac{B_X}{[\mathrm{X}]} \tag{S13}$$

The differential $d\bar{N}_X/d[\mathrm{X}]$ is equivalent to the differential $d\beta/dC_S$ employed by Lapitsky et al.[3] for the calorimetry titration of a polyelectrolyte with a surfactant molecule, where the term β represents the average fractional coverage of the binding sites of polyelectrolyte based on the Satake-Yang model, and C_S represents the free concentration of surfactant. In addition,

Vinnakota et. al. evaluated the differential $d\bar{N}_X / d[\mathrm{X}]$ numerically for the analysis of pH dependent enzymatic reactions, such as muscle glycogenolysis.[4]

The differential $d\alpha_i / d\Phi$ represents the infinitesimal change in the mole fraction of a receptor bound to *i* ligands with respect to the degree of titration Φ, and is calculated using the chain rule as given by

$$\frac{d\alpha_i}{d\Phi} = \frac{d\alpha_i}{d[\mathrm{X}]} \cdot \frac{d[\mathrm{X}]}{d\Phi} \tag{S14}$$

where the term $d\alpha_i / d[\mathrm{X}]$ is obtained from S5 as follows

$$\begin{aligned} \bar{F}_i &= \frac{d \ln \alpha_i}{d \ln[\mathrm{X}]} \\ &= \frac{[\mathrm{X}]}{\alpha_i} \cdot \frac{d\alpha_i}{d[\mathrm{X}]} \end{aligned} \tag{S15}$$

thus,

$$\begin{aligned} \frac{d\alpha_i}{d[\mathrm{X}]} &= \bar{F}_i \cdot \frac{\alpha_i}{[\mathrm{X}]} \\ &= (i - \bar{N}_X) \cdot \frac{\alpha_i}{[\mathrm{X}]} \end{aligned} \tag{S16}$$

The final expression for $d\alpha_i / d\Phi$ is obtained by combining eqs S11 and S16 to give

$$\frac{d\alpha_i}{d\Phi} = \frac{M_T \alpha_i (i - \bar{N}_X)}{[\mathrm{X}] + M_T B_X} \tag{S17}$$

Finally, the differential $d\bar{N}_X / d\Phi$ represents the differential change in average number of ligand species bound to a receptor with respect to Φ and is calculated using eqs S8 and S11. First, the mass balance equation is rearranged

$$\bar{N}_X = \Phi - \frac{[\mathrm{X}]}{M_T} \tag{S18}$$

Then, differentiation with respect to Φ at constant M_T gives

$$\begin{aligned}\frac{d\bar{N}_X}{d\Phi} &= 1-\frac{1}{M_T}\cdot\frac{d[\mathrm{X}]}{d\Phi} \\ &= 1-\frac{[\mathrm{X}]}{[\mathrm{X}]+M_T B_X} \\ &= \frac{M_T B_X}{[\mathrm{X}]+M_T B_X}\end{aligned} \tag{S19}$$

Incidentally, $d\bar{N}_X / d\Phi$ is also equivalent to the i weighted sum of the differentials $d\alpha_i / d\Phi$. The relation between $d\bar{N}_X / d\Phi$ and $d\alpha_i / d\Phi$ is obtained using the relation

$$\bar{N}_X = \sum_{i=0}^{n} i\cdot\alpha_i \tag{S20}$$

By differentiating of eq S20 with respect to Φ, we obtain

$$\begin{aligned}\frac{d\bar{N}_X}{d\Phi} &= \sum_{i=0}^{n}\left(i\cdot\frac{d\alpha_i}{d\Phi}\right)=\sum_{i==}^{n}\left(\frac{i\cdot M_T\alpha_i(i-\bar{N}_X)}{[\mathrm{X}]+M_T B_X}\right) \\ &= \frac{M_T}{[\mathrm{X}]+M_T B_X}\sum_{i=0}^{n}(i^2\alpha_i-\bar{N}_X\cdot i\alpha_i)\end{aligned} \tag{S21a}$$

After substitution of the explicit form of the mole fraction in terms of the cumulative binding constants β_i and P_M (eq S4), we obtain

$$\begin{aligned}\frac{d\bar{N}_X}{d\Phi} &= \frac{M_T}{[\mathrm{X}]+M_T B_X}\left(\frac{\sum_{i=0}^{n} i^2\beta_i[\mathrm{X}]^i}{P_M}-\bar{N}_X\cdot\frac{\sum_{i=0}^{n} i\beta_i[\mathrm{X}]^i}{P_M}\right) \\ &= \frac{M_T}{[\mathrm{X}]+M_T B_X}\left(\frac{\sum_{i=0}^{n} i^2\beta_i[\mathrm{X}]^i}{P_M}-\left(\frac{\sum_{i=0}^{n} i\beta_i[\mathrm{X}]^i}{P_M}\right)^2\right) \\ &= \frac{M_T B_X}{[\mathrm{X}]+M_T B_X}\end{aligned} \tag{S21b}$$

where the first summation term inside the parenthesis is proportional to the second moment of P_M and the second summation term is proportional to the square of the first moment of P_M. The summation terms for the first moment and the second moment of P_M are equivalent the binding capacity B_X (eq S3b).

The general form of the DBM that describes the rate of change in the receptor normalized concentrations of the binding species in solution as well as the overall change the binding saturation is given by eqs S11, S17, and S19. Thus, the set of model-independent differential equations in the general DBM can be written as

$$\left(\frac{1}{M_T}\right)\cdot\frac{d[\mathrm{X}]}{d\Phi} = \frac{[\mathrm{X}]}{[\mathrm{X}]+M_T B_X} \tag{S22a}$$

$$\frac{d\alpha_i}{d\Phi} = \frac{M_T\alpha_i(i-\bar{N}_X)}{[\mathrm{X}]+M_T B_X} \tag{S22b}$$

$$\frac{d\bar{N}_X}{d\Phi} = \frac{M_T B_X}{[\mathrm{X}]+M_T B_X} \tag{S22c}$$

The differential heat per mole *dH* is given by the cumulative heat per mole contribution for all the binding species in solution. The general expression to evaluate the differential heat per mole in terms of the total concentration of ligand injected to the titration cell X_T is given by

$$dH = \sum_{i=1}^{n}\Delta H_i\frac{d[\mathrm{MX}_i]}{dX_T} \tag{S23}$$

The expression for *dH* in terms of the degree of titration (Φ) is derived as follows. First, we use the chain rule to express $d[\mathrm{MX}_i]/dX_T$ in terms of Φ, as given by

$$\frac{d[\mathrm{MX}_i]}{dX_T} = \frac{d\Phi}{dX_T}\cdot\frac{d[\mathrm{MX}_i]}{d\Phi} \tag{S24a}$$

and after using the relation $d\Phi = dX_T/M_T$ for a titration at constant M_T, we obtain

$$\frac{d[\mathrm{MX}_i]}{dX_T} = \frac{1}{M_T}\cdot\frac{d[\mathrm{MX}_i]}{d\Phi} \tag{S24b}$$

Similarly, we can use the relation $d[\mathrm{MX}_i] = M_T\cdot d\alpha_i$ in eq S24b, which gives

$$\frac{d[\mathrm{MX}_i]}{dX_T} = \frac{M_T}{M_T}\cdot\frac{d\alpha_i}{d\Phi} = \frac{d\alpha_i}{d\Phi} \tag{S24c}$$

Thus, the final expression for *dH* in terms of Φ is given by

$$dH = \sum_{i=1}^{n} \Delta H_i \frac{d\alpha_i}{d\Phi} \quad \text{(S25)}$$

The previous expression of dH (eqs S23 and S25) represent an idealized calorimetry titration where the concentrations of the binding species are accurately known and where the heat contribution due to the dilution of the ligand or its interaction with the buffer is nearly zero ($dH_{dil} \approx 0$). First, it is possible to include an adjustable parameter to account for the enthalpy of dilution as given by

$$dH = \sum_{i=1}^{n} \Delta H_i \frac{d\alpha_i}{d\Phi} + \Delta h_{dil} \quad \text{(S26)}$$

where Δh_{dil} is a constant value equal to the dH signal of the final 3-5 injection points in the titration curve and the term Δh_{dil} has the effect of shifting the fitting curve vertically. This approach assumes that the heat values of final injection points are nearly constant and that the titration of a ligand solution into the background buffer also gives a constant value. However, if the final injection points do not show a constant value, we can derive an expression that takes into account the change in the concentration of free ligand in solution as shown below.

The enthalpy of dilution for a titration where the change in the free ligand concentration contributes is represented as

$$dH_{dil} = \frac{\Delta h_d}{M_T} \cdot \frac{d[\mathrm{X}]}{d\Phi} \quad \text{(S27)}$$

which was obtained from the differential $d[\mathrm{X}]/dX_T$ after following similar derivation steps as those used to obtain the differential $d[\mathrm{MX}_i]/d\Phi$ (eqs S24a-S24c). The expression of dH that accounts for the binding equilibrium reactions as well as the dilution of free ligand is given by

$$dH = \sum_{i=1}^{n} \Delta H_i \frac{d\alpha_i}{d\Phi} + \frac{\Delta h_d}{M_T} \cdot \frac{d[\mathrm{X}]}{d\Phi} \quad \text{(S28)}$$

and the explicit expression for S28 in terms of the binding potentials is given by

$$dH = \frac{M_T \cdot \sum_{i=1}^{n} \Delta H_i \cdot \alpha_i (i - \bar{N}_X) + \Delta h_d \cdot [\mathrm{X}]}{[\mathrm{X}] + M_T B_X} \quad \text{(S29)}$$

Second, we can include a stoichiometry coefficient to adjust for small errors in the concentration of the binding species. Typically, the total concentration of receptor M_T is the parameter that is adjusted during the analysis of a "direct" titration where ligand is injected into a receptor solution.[5,6] Hence, the M_T terms in eq S29 can be replaced by an adjusted receptor concentration $M_T^* = N \cdot M_T$, where N represents the stoichiometry coefficient. The expression to evaluate dH that accounts for both small errors in the concentration of M_T as well as the enthalpy of dilution for the ligand is given by

$$dH = \frac{N \cdot M_T \sum_{i=1}^{n} \Delta H_i \cdot \alpha_i (i - \bar{N}_X) + \Delta h_d \cdot [\mathrm{X}]}{[\mathrm{X}] + N \cdot M_T B_X} \tag{S30}$$

The total concentration of ligand X_T may also be adjusted for a direct titration, but this would require that the dH points in the titration curve were recalculated at every minimization step, as given by

$$dH = \frac{1}{N \cdot V_C} \cdot \frac{dq}{dX_T} \tag{S31}$$

However, the terms X_T and M_T cannot be both adjusted simultaneously because this over-parameterizes the binding model.

B. DBM for a Receptor with *One* Binding Site ($n = 1$).

1. *Binding Potentials and General Binding Model*

The binding polynomial for a receptor with *one* binding site is given by

$$P_M = 1 + K[\mathrm{X}] \tag{S32}$$

where K is the binding constant for the equilibrium reaction

$$\mathrm{M} + \mathrm{X} \rightleftarrows \mathrm{MX} \quad , \quad K = \frac{[\mathrm{MX}]}{[\mathrm{M}][\mathrm{X}]} \tag{S33}$$

with the mass balance equations

$$\begin{aligned} X_T &= [\mathrm{X}] + [\mathrm{MX}] \\ M_T &= [\mathrm{M}] + [\mathrm{MX}] \end{aligned} \tag{S34}$$

The mole fraction for the free ($\alpha_0 = [\mathrm{M}]/M_T$) and bound receptor $\alpha_1 = [\mathrm{MX}]/M_T$ in terms of the binding polynomial are given respectively by

$$\alpha_0 = \frac{1}{P_M} \quad , \quad \alpha_1 = \frac{K[\mathrm{X}]}{P_M} \tag{S35}$$

and the binding potentials $\bar{N}_X$ and B_X are given by

$$\bar{N}_X = \frac{K[\mathrm{X}]}{P_M} \quad , \quad B_X = \frac{K[\mathrm{X}]}{(P_M)^2} \tag{S36}$$

Substitution of the binding potentials $\bar{N}_X$ and B_X, and the mole fractions α_0 and α_1 into eqs S22a-S22c gives

$$\frac{1}{M_T}\cdot\frac{d[\mathrm{X}]}{d\Phi} = \frac{(1+K[\mathrm{X}])^2}{M_T K + (1+K[\mathrm{X}])^2} = \frac{{P_M}^2}{c+{P_M}^2} \tag{S37a}$$

$$\frac{d\alpha_0}{d\Phi} = -\frac{M_T K}{M_T K + (1+K[\mathrm{X}])^2} = -\frac{c}{c+{P_M}^2} \tag{S37b}$$

$$\frac{d\alpha_1}{d\Phi} = \frac{M_T K}{M_T K + (1+K[\mathrm{X}])^2} = \frac{c}{c+{P_M}^2} \tag{S37c}$$

We write the term M_T on the left hand side of eq S37a in order to make this expression dimensionless. In addition, we can observe that the differential equations in the DBM (eqs S37a-S37c) have a common denominator given by the term $c+{P_M}^2$.

2. *Theoretical Evaluation of the DBM*

The term $K[\mathrm{X}]$ can be expressed in terms of the average ligand saturation $\bar{N}_X$ and the binding constant, as given by

$$K[\mathrm{X}] = \left(\frac{\bar{N}_X}{1-\bar{N}_X}\right) \tag{S38}$$

After substitution of eq S38 into the general expression for the mass balance equation (eq S8), we obtain an expression for the degree of titration in terms of $\bar{N}_X$ and the dimensionless parameter $c = K{\cdot}M_T$

$$\Phi = \bar{N}_X + \frac{1}{c}\cdot\left(\frac{\bar{N}_X}{1-\bar{N}_X}\right) \tag{S39}$$

Thus, the degree of titration (Φ) can be evaluated as a function of the average number of ligand molecules per receptor and the parameter *c*. The degree of titration Φ required to saturate 10%, 50%, and 90% can be calculated using $\bar{N}_X$ values of 1/11, 1/2, and 10/11, respectively, to obtain

$$\Phi^{10\%} = \frac{1}{11}+\frac{1}{10c} \tag{S40a}$$

$$\Phi^{50\%} = \frac{1}{2}+\frac{1}{c} \tag{S40b}$$

$$\Phi^{90\%} = \frac{10}{11}+\frac{10}{c} \tag{S40c}$$

Similarly, the expression for the differential binding $d\bar{N}_X / d\Phi$ (eq 22) can be evaluated as a function of $\bar{N}_X$. The values for the differential $d\bar{N}_X / d\Phi$ at 10%, 50%, and 90% saturation correspond to

$$\left.\frac{d\bar{N}_X}{d\Phi}\right|_{10\%} = \frac{c}{c+(1/1.21)} \tag{S41a}$$

$$\left.\frac{d\bar{N}_X}{d\Phi}\right|_{50\%} = \frac{c}{c+4} \tag{S41b}$$

$$\left.\frac{d\bar{N}_X}{d\Phi}\right|_{90\%} = \frac{c}{c+121} \tag{S41c}$$

3. *Numerical Evaluation of the DBM*

In order to analyze an ITC experiment with the DBM in eqs S37a-c, we need an expression to calculate the concentration of free ligand [X] at every injection step. For this purpose, we derive an exact algebraic expression to evaluate [X] as a function of the terms of Φ and *c*. The mass balance expression for a receptor with one binding site in terms of Φ is given by

$$\Phi = \frac{[\mathrm{X}]}{M_T}+\frac{K[\mathrm{X}]}{1+K[\mathrm{X}]} \tag{S42}$$

After rearrangement of eq S42, we obtain the quadratic polynomial

$$[\mathrm{X}]^2 \cdot \frac{1}{M_T} + [\mathrm{X}]\left(1 + \frac{1}{M_T K} - \Phi\right) - \frac{\Phi}{K} = 0 \tag{S43}$$

with a positive root given by

$$[\mathrm{X}] = M_T \cdot \frac{\Phi - \frac{1}{c} - 1 + \left(\left(1 + \frac{1}{c} - \Phi\right)^2 + \frac{4\Phi}{c}\right)^{1/2}}{2} \tag{S44}$$

and multiplication of both sides by K gives

$$K[\mathrm{X}] = \frac{c(\Phi - 1) - 1 + \left(\left(1 - c(\Phi - 1)\right)^2 + 4c\Phi\right)^{1/2}}{2} \tag{S45}$$

We can use eq S45 to evaluate the binding polynomial P_M (eq S32) and the DBM (eqs S37a-c) at specific values of Φ and c. For instance, the expression for the binding polynomial P_M in terms of Φ and c is given by

$$P_M = \frac{1 + c(\Phi - 1) + \left(\left(1 - c(\Phi - 1)\right)^2 + 4c\Phi\right)^{1/2}}{2} \tag{S46}$$

In addition, we can evaluate P_M and $d\alpha_1/d\Phi$ at specific points on the titration curve. For example, the value of P_M at the equivalence point ($\Phi = 1.0$) is

$$P_{M,\Phi=1.0} = \frac{1 + (1 + 4c)^{1/2}}{2} \tag{S47}$$

and the differential $d\alpha_1/d\Phi$ corresponds to

$$\left.\frac{da_1}{d\Phi}\right|_{\Phi=1.0} = \frac{2c}{1 + 4c + (1 + 4c)^{1/2}} \tag{S48}$$

which approaches 0.5 in the limit of $c \rightarrow \infty$, as previously shown by Poon.[10]

The previous expressions (S44-S48) are based on the assumption that the terms M_T and c remain constant during the titration. To account the dilution and overflow of the binding species during the titration in a calorimeter with an overfill-cell, we evaluate the terms X_T, M_T, and Φ with the expressions.

$$X_T = X_S \cdot (1 - \exp(-V / V_C)) \tag{S49a}$$

$$M_T = M_0 \cdot \exp(-V / V_C) \tag{S49b}$$

$$\Phi = X_S \cdot (\exp(V / V_C) - 1) / M_0 \tag{S49c}$$

where X_S is the concentration of ligand in the syringe, M_0 is the initial concentration of receptor in the cell, V_C is the cell volume, and V represents the total volume of titrant injected. Finally, to analyze an ITC experiment, one can write an algorithm that combines the dilution expressions for the binding species (S49a-S49c), the exact algebraic solution for K[X] (eq S45), and the DBM (eqs S37a-S37c) with non-linear regression routine.

C. DBM for a Receptor with *Two* Binding Sites (n = 2)

1. *Binding Potentials and General Binding Model*

The binding polynomial for a receptor with *two* binding sites is given by

$$P_M = 1 + \beta_1[\mathrm{X}] + \beta_2[\mathrm{X}]^2 \tag{S50}$$

where β_1 and β_2 are the binding constants for the equilibrium reactions

$$\mathrm{M} + \mathrm{X} \rightleftarrows \mathrm{MX} \quad , \quad \beta_1 = \frac{[\mathrm{MX}]}{[\mathrm{M}][\mathrm{X}]} \tag{S51a}$$

$$\mathrm{M} + 2\mathrm{X} \rightleftarrows \mathrm{MX_2} \quad , \quad \beta_2 = \frac{[\mathrm{MX_2}]}{[\mathrm{M}][\mathrm{X}]^2} \tag{S51b}$$

with the mass balance equations

$$\begin{aligned} X_T &= [\mathrm{X}] + [\mathrm{MX}] + 2[\mathrm{MX_2}] \\ M_T &= [\mathrm{M}] + [\mathrm{MX}] + [\mathrm{MX_2}] \end{aligned} \tag{S52}$$

The mole fraction for the free ($\alpha_0 = [\mathrm{M}] / M_T$) and bound receptor species ($\alpha_1 = [\mathrm{MX}] / M_T, \alpha_2 = [\mathrm{MX_2}] / M_T$) in terms of the binding polynomial are given respectively by

$$\alpha_0 = \frac{1}{P_M} \quad , \quad \alpha_1 = \frac{\beta_1[\mathrm{X}]}{P_M} \quad , \quad \alpha_2 = \frac{\beta_2[\mathrm{X}]^2}{P_M} \tag{S53}$$

and the binding potentials $\bar{N}_X$ and B_X are given by

$$\bar{N}_X = \frac{\beta_1[\mathrm{X}] + 2\beta_2[\mathrm{X}]^2}{P_M} \tag{S54a}$$

$$B_X = \frac{\beta_1[\mathrm{X}] + 4\beta_2[\mathrm{X}]^2 + \beta_1\beta_2[\mathrm{X}]^3}{(P_M)^2} \tag{S54b}$$

We obtain the set of model-independent differential equations (i.e., general DBM) by substituting the binding potentials in eqs S22a-S22c as given by

$$\frac{1}{M_T}\cdot\frac{d[\mathrm{X}]}{d\Phi} = \frac{{P_M}^2}{{P_M}^2 + M_T(\beta_1 + 4\beta_2[X] + \beta_1\beta_2[X]^2)} \tag{S55a}$$

$$\frac{d\alpha_0}{d\Phi} = -\frac{M_T(\beta_1 + 2\beta_2[X])}{{P_M}^2 + M_T(\beta_1 + 4\beta_2[X] + \beta_1\beta_2[X]^2)} \tag{S55b}$$

$$\frac{d\alpha_1}{d\Phi} = \frac{M_T\beta_1(1 - \beta_2[X]^2)}{{P_M}^2 + M_T(\beta_1 + 4\beta_2[X] + \beta_1\beta_2[X]^2)} \tag{S55c}$$

$$\frac{d\alpha_2}{d\Phi} = \frac{M_T\beta_2[\mathrm{X}](2 + \beta_1[X])}{{P_M}^2 + M_T(\beta_1 + 4\beta_2[X] + \beta_1\beta_2[X]^2)} \tag{S55d}$$

$$\frac{d\bar{N}_X}{d\Phi} = \frac{M_T(\beta_1 + 4\beta_2[X] + \beta_1\beta_2[X]^2)}{{P_M}^2 + M_T(\beta_1 + 4\beta_2[X] + \beta_1\beta_2[X]^2)} \tag{S55e}$$

where

$$\frac{d\alpha_0}{d\Phi} + \frac{d\alpha_1}{d\Phi} + \frac{d\alpha_2}{d\Phi} = 0 \tag{S56}$$

,and

$$\frac{d\bar{N}_X}{d\Phi} = \frac{d\alpha_1}{d\Phi} + 2\cdot\frac{d\alpha_2}{d\Phi} \tag{S57}$$

The expression to evaluate dH using the differentials $d\alpha_2/d\Phi$ and $d\alpha_2/d\Phi$ (eqs S55c and S55d) and the cumulative enthalpies of reaction ΔH_1, ΔH_2 is given by

$$\begin{aligned} dH &= \Delta H_1\frac{d\alpha_1}{d\Phi} + \Delta H_2\frac{d\alpha_2}{d\Phi} \\ &= M_T\cdot\frac{\Delta H_1\cdot\beta_1(1 - \beta_2[X]^2) + \Delta H_2\cdot\beta_2[\mathrm{X}](2 + \beta_1[X])}{{P_M}^2 + M_T(\beta_1 + 4\beta_2[X] + \beta_1\beta_2[X]^2)} \end{aligned} \tag{S58}$$

Similar to the DBM for a receptor with multiple binding sites (S30), the general expression to evaluate dH with $n = 2$ can be extended to account for both the dilution of the free ligand [X], and small errors in the concentration of receptor M_T as given by

$$dH = \frac{N \cdot M_T \left(\Delta H_1 \cdot \beta_1 (1 - \beta_2 [X]^2) + \Delta H_2 \cdot \beta_2 [\mathrm{X}](2 + \beta_1 [X]) \right) + \Delta h_{dil} {P_M}^2}{{P_M}^2 + N \cdot M_T (\beta_1 + 4\beta_2 [X] + \beta_1 \beta_2 [X]^2)} \tag{S59}$$

The limiting value of *dH* at the beginning of the titration for eq S58 (i.e., $\Phi \rightarrow 0$) after evaluation with the term [X] = 0, correspond to

$$dH|_{[\mathrm{X}]=0} \quad = \quad \Delta H_1 \cdot \frac{M_T \beta_1}{1 + M_T \beta_1} \quad = \quad \frac{\Delta H_1 \cdot c_0}{1 + c_0} \tag{S60}$$

where the dimensionless parameter c_0 is equal to the product $M_T \cdot \beta_1$. The term c_0 is similar to the parameter *c* in eqs S37a-S37c, and it can be used to optimize the initial value of *dH* in a titration experiment for a receptor with two binding sites.

2. *Macroscopic Binding Model: Sequential Binding Sites*

The binding polynomial for a receptor with *two* binding sites and sequential binding is given by

$$P_M = 1 + K_1[\mathrm{X}] + K_1 K_2 [\mathrm{X}]^2 \tag{S61}$$

where K_1 and K_2 are the binding constant for the stepwise equilibrium reactions

$$\mathrm{M} + \mathrm{X} \rightleftarrows \mathrm{MX} \quad , \quad K_1 = \frac{[\mathrm{MX}]}{[\mathrm{M}][\mathrm{X}]} \tag{S62a}$$

$$\mathrm{MX} + \mathrm{X} \rightleftarrows \mathrm{MX_2} \quad , \quad K_2 = \frac{[\mathrm{MX_2}]}{[\mathrm{MX}][\mathrm{X}]} \tag{S62b}$$

and the enthalpy of binding for each stepwise equilibrium reaction is given by ΔH_1^0 and ΔH_2^1, respectively. The mole fractions for the free and bound receptor species are given respectively by

$$\alpha_0 = \frac{1}{P_M} \quad , \quad \alpha_1 = \frac{K_1[\mathrm{X}]}{P_M} \quad , \quad \alpha_2 = \frac{K_1 K_2 [\mathrm{X}]^2}{P_M} \tag{S63}$$

and the binding potentials $\bar{N}_X$ and B_X are given by

$$\bar{N}_X = \frac{K_1[\mathrm{X}](1 + 2K_2[\mathrm{X}])}{P_M} \quad , \quad B_X = \frac{K_1[\mathrm{X}](1 + 4K_2[\mathrm{X}] + K_1 K_2 [\mathrm{X}]^2)}{(P_M)^2} \tag{S64}$$

After substitution of the binding potentials $\bar{N}_X$ and B_X into eqs S55c and S55d , we obtain

$$\frac{d\alpha_1}{d\Phi} = \frac{M_T K_1 (1 - K_1 K_2 [X]^2)}{P_M{}^2 + M_T K_1 \left(1 + 4K_2[X] + K_1 K_2 [X]^2\right)} \quad \text{(S65a)}$$

$$\frac{d\alpha_2}{d\Phi} = \frac{M_T K_1 K_2 [\mathrm{X}](2 + K_1[X])}{P_M{}^2 + M_T K_1 \left(1 + 4K_2[X] + K_1 K_2 [X]^2\right)} \quad \text{(S65b)}$$

Finally, the differential heat per mole is evaluated with the expression

$$dH = \Delta H_1^0 \frac{d\alpha_1}{d\Phi} + (\Delta H_1^0 + \Delta H_2^1) \frac{d\alpha_2}{d\Phi} \quad \text{(S66)}$$

After substitution of eqs S65a and S65b in eq S66, we obtain

$$dH = \frac{M_T K_1 \left(\Delta H_1^0 \cdot \left(1 + 2K_1 K_2 [X]\right) + \Delta H_2^1 \left(2K_2 [\mathrm{X}] + K_1 K_2 [\mathrm{X}]^2\right)\right)}{P_M{}^2 + M_T K_1 \left(1 + 4K_2[X] + K_1 K_2 [X]^2\right)} \quad \text{(S67)}$$

The limiting value of the term *dH* (eq S67) at [X] = 0 is equivalent to the expression for *one* binding site (eq 21) evaluated at [X]=0.

$$dH|_{[\mathrm{X}]=0} = \Delta H_1^0 \cdot \frac{M_T K_1}{1 + M_T K_1} \quad \text{(S68)}$$

The term *dH* for a receptor with sequential binding sites can be also evaluated at concentrations of free ligand that simplify some of the terms in eq S67, such as [X] = $1/K_1$, $1/K_2$, or $1/(K_1 K_2)^{1/2}$.

3. *Microscopic Binding Model: Independent Binding Sites*

The binding polynomial for a receptor with two independent binding sites is expressed as the product of the partition functions for each microscopic site (i.e., $P_{M(1)}$, and $P_{M(2)}$) as given by

$$P_M = P_{M(1)} \cdot P_{M(2)} \quad \text{(S69)}$$

The partition function for each microscopic site is given by

$$P_{M(i)} = \sum_{j=0}^{j=1} k_{ij} [\mathrm{X}]^j \quad \text{(S70)}$$

where k_{ij} is the microscopic binding constant, the sub-index *i* represents the site number, and the sub-index *j* represents the occupancy of the binding level. The microscopic constants for the unbound states are defined as $k_{10} = k_{20} = 1$. The general expression to calculate the mole fraction of the bound and free binding states for each microscopic site is

$$\alpha_{i0} = \frac{1}{P_{M(i)}} \quad , \quad \alpha_{i1} = \frac{k_{i1}[\mathrm{X}]}{P_{M(i)}} \quad \text{(S71)}$$

The total average number of ligands per receptor species $\bar{N}_X$ is calculated using the differential expression in eq S3a as follows

$$\begin{aligned} \bar{N}_X &= \frac{d\ln P_M}{d\ln[\mathrm{X}]} \\ &= \frac{d\ln(P_{M(1)})}{d\ln[\mathrm{X}]} + \frac{d\ln(P_{M(2)})}{d\ln[\mathrm{X}]} \\ &= \frac{k_{11}[\mathrm{X}]}{1+k_{11}[\mathrm{X}]} + \frac{k_{21}[\mathrm{X}]}{1+k_{21}[\mathrm{X}]} \\ &= \bar{N}_{X(1)} + \bar{N}_{X(2)} \end{aligned} \tag{S72}$$

where $\bar{N}_{X(i)}$ corresponds to the average saturation for the *i*-th microscopic site. Using the same formalism, we obtain an expression for the total binding capacity in terms of each microscopic binding capacity ($B_{X(i)}$), as given by

$$\begin{aligned} B_X &= \frac{d^2\ln P_M}{d(\ln[\mathrm{X}])^2} \\ &= \frac{d^2\ln(P_{M(1)})}{d(\ln[\mathrm{X}])^2} + \frac{d^2\ln(P_{M(2)})}{d(\ln[\mathrm{X}])^2} \\ &= \frac{k_{11}[\mathrm{X}]}{(1+k_{11}[\mathrm{X}])^2} + \frac{k_{21}[\mathrm{X}]}{(1+k_{21}[\mathrm{X}])^2} \\ &= B_{X(1)} + B_{X(2)} \end{aligned} \tag{S73}$$

The resulting binding potentials $\bar{N}_X$ and B_X are substituted into eqs S22a-S22c which gives

$$\begin{aligned} \frac{1}{M_T}\cdot\frac{d[\mathrm{X}]}{d\Phi} &= \frac{1}{1+\frac{M_T k_{11}}{(P_{M(1)})^2}+\frac{M_T k_{21}}{(P_{M(2)})^2}} \\ &= \frac{1}{1+\frac{c_{(1)}}{(P_{M(1)})^2}+\frac{c_{(2)}}{(P_{M(2)})^2}} \end{aligned} \tag{S74a}$$

$$\frac{d\alpha_{10}}{d\Phi} = -\frac{d\alpha_{11}}{d\Phi} \tag{S74b}$$

$$\frac{d\alpha_{11}}{d\Phi} = \frac{\dfrac{M_T k_{11}}{(P_{M(1)})^2}}{1+\dfrac{M_T k_{11}}{(P_{M(1)})^2}+\dfrac{M_T k_{21}}{(P_{M(2)})^2}} = \frac{c_{(1)}}{c_{(1)}+(P_{M(1)})^2\left(1+\dfrac{c_{(1)}}{(P_{M(2)})^2}\right)} \quad \text{(S74c)}$$

$$\frac{d\alpha_{20}}{d\Phi} = -\frac{d\alpha_{21}}{d\Phi} \quad \text{(S74d)}$$

$$\frac{d\alpha_{21}}{d\Phi} = \frac{\dfrac{M_T k_{21}}{(P_{M(1)})^2}}{1+\dfrac{M_T k_{11}}{(P_{M(1)})^2}+\dfrac{M_T k_{21}}{(P_{M(2)})^2}} = \frac{c_{(2)}}{c_{(2)}+(P_{M(2)})^2\left(1+\dfrac{c_{(1)}}{(P_{M(1)})^2}\right)} \quad \text{(S74e)}$$

where the differential heat per mole is evaluated with eq 24a

$$dH = \Delta h_1 \frac{d\alpha_{11}}{d\Phi} + \Delta h_2 \frac{d\alpha_{21}}{d\Phi} \quad \text{(S75)}$$

The additive property of the binding potentials $\bar{N}_{X(i)}$ and $B_{X(i)}$ allows for each microscopic binding site allow us to expand this model to a receptor with *n*-independent binding sites (see eqs 35a and 35b, main text).

4. *Microscopic Binding Model: Equivalent Binding Sites*

If the microscopic binding constants for the same level of occupancy are equivalent (i.e., $k_{10} = k_{20} = 1$, and $k_{11} = k_{21} = k$), then the expression for the binding polynomial (eq S69) reduces to

$$P_M = (P_{eq})^2 = (1+k[\mathrm{X}])^2 \quad \text{(S76)}$$

where k is the intrinsic binding constant for the bound species. Similarly, the differentials shown in eqs S74a-S74e reduce to

$$\frac{1}{M_T}\frac{d[\mathrm{X}]}{d\Phi} = \frac{(P_{eq})^2}{(P_{eq})^2+2M_T k} = \frac{(P_{eq})^2}{(P_{eq})^2+2c_{eq}} \quad \text{(S77a)}$$

$$\frac{d\alpha_{10}}{d\Phi} = -\frac{d\alpha_{11}}{d\Phi} \quad \text{(S77b)}$$

$$\frac{d\alpha_{11}}{d\Phi} = \frac{M_T k}{(P_{eq})^2 + 2M_T k} = \frac{c_{eq}}{(P_{eq})^2 + 2c_{eq}} \quad \text{(S77c)}$$

where c_{eq} is the dimensionless binding parameter for the titration of a receptor with equivalent binding sites, proportional to the product of the intrinsic binding constant *k* and total receptor concentration M_T (i.e., $c_{eq} = M_T \cdot k$). If the enthalpies of binding of each independent binding site are equivalent (i.e., $\Delta h_{11} = \Delta h_{21} = \Delta h$), then the differential heat per mole *dH* for a receptor with two identical binding sites can be evaluated with the expression

$$dH = 2 \cdot \Delta h \frac{d\alpha_{11}}{d\Phi} = \Delta h \cdot \frac{2c_{eq}}{(P_{eq})^2 + 2c_{eq}} \quad \text{(S78)}$$

5. *Theoretical Evaluation: DBM with Cooperative Binding Interactions*

We use the concentration of free ligand at 50% fractional binding saturation (i.e., $\bar{N}_X / n$) to evaluate the DBM for a receptor with two binding sites (*n* = 2). Here, we follow a similar procedure to that used in the case of a receptor with *n* = 1. The 50% fractional binding saturation corresponds to one ligand bound per receptor (i.e., $\bar{N}_X$ = 1). Using eq S54a, we calculate the free ligand concentration at 50% saturation ($[\mathrm{X}]^{50\%}$) in terms of the cumulative binding constant(s), which corresponds to $[\mathrm{X}]^{50\%} = (\beta_2)^{-1/2}$. Thus, we can use the dimensionless variable $\beta_2^{1/2}[\mathrm{X}]$ to evaluate the binding potentials $\bar{N}_X$, B_X, the degree of titration Φ, and the DBM in eqs S55a-S55c.

The general expression to evaluate the degree of titration Φ of a receptor with multiple binding sites is given in eq S8. In order to make this expression compatible with the dimensionless variable $\beta_2^{1/2}[\mathrm{X}]$, we can multiply both the numerator and the denominator in the term $[\mathrm{X}]/M_T$ by $\beta_2^{1/2}$, as given by

$$\Phi = \bar{N}_X + \frac{\beta_2^{1/2}[X]}{c_{1/2}} \tag{S79}$$

where $c_{1/2}$ is a constant dimensionless parameter for the titration

$$c_{1/2} = M_T \cdot \beta_2^{1/2} \propto M_T / [\mathrm{X}]^{50\%} \tag{S80}$$

proportional to the ratio between the total receptor concentration and the concentration of free ligand at half-saturation. In addition, the dimensionless variable $\beta_2^{1/2}[\mathrm{X}]$ in eq S79 can be expressed in terms of $\bar{N}_X$ using eq S54a and the algebraic solution to a quadratic polynomial, as given by

$$\beta_2^{1/2}[X] = \frac{\beta_1}{2\beta_2^{1/2}} \cdot \frac{\bar{N}_X - 1}{2 - \bar{N}_X} + \left(\frac{\beta_1^2}{4\beta_2} \cdot \left(\frac{\bar{N}_X - 1}{2 - \bar{N}_X} \right)^2 + \frac{\bar{N}_X}{2 - \bar{N}_X} \right)^{1/2} \tag{S81}$$

Interestingly, the term $\beta_1 / (2\beta_2^{1/2})$ is inversely proportional to the square root of the stepwise cooperativity parameter ρ. Thus, eqs S79, and S81 can be rewritten to include the term ρ

$$\Phi = \bar{N}_X + \frac{c_\rho}{c_{1/2}} \tag{S82}$$

where c_ρ is the dimensionless variable expressed in terms of ρ and $\bar{N}_X$, as given by.

$$c_\rho = \frac{1}{\rho^{1/2}} \left(\frac{\bar{N}_X - 1}{2 - \bar{N}_X} + \left(\left(\frac{\bar{N}_X - 1}{2 - \bar{N}_X} \right)^2 + \rho \cdot \frac{\bar{N}_X}{2 - \bar{N}_X} \right)^{1/2} \right) \tag{S83}$$

The cooperativity variable c_ρ at 10%, 50%, and 90% saturation can be evaluated using the values of $\bar{N}_X$ equal to 2/11, 1.0, and 20/11, respectively as given by

$$c_\rho^{10\%} = \frac{1}{\rho^{1/2}} \left(-\frac{9}{20} + \left(\left(\frac{9}{20} \right)^2 + \frac{\rho}{10} \right)^{1/2} \right) \tag{S84a}$$

$$c_\rho^{50\%} = 1 \tag{S84b}$$

$$c_\rho^{90\%} = \frac{1}{\rho^{1/2}} \left(\frac{9}{2} + \left(\left(\frac{9}{2} \right)^2 + 10\rho \right)^{1/2} \right) \tag{S84c}$$

Thus, the degrees of titration required to reach 10%, 50%, and 90% saturation are given respectively by

$$\Phi^{10\%} = \frac{2}{11} + \frac{c_\rho^{10\%}}{c_{1/2}} \quad \text{(S85a)}$$

$$\Phi^{50\%} = 1 + \frac{1}{c_{1/2}} \quad \text{(S85b)}$$

$$\Phi^{90\%} = \frac{20}{11} + \frac{c_\rho^{90\%}}{c_{1/2}} \quad \text{(S85c)}$$

A similar strategy can be used to evaluate the differentials in the DBM for a receptor with n = 2 (eqs S55a-S55e). However, we only evaluate the terms $d\alpha_1 / d\Phi$ and $d\alpha_2 / d\Phi$ because they show interesting properties in relation to the fractional saturation $\bar{N}_X / n$. In addition, the terms $d\alpha_1 / d\Phi$ and $d\alpha_2 / d\Phi$ are the main contribution to the differential heat per mole dH (eq S58). For example, the limiting values of $d\alpha_1 / d\Phi$ and $d\alpha_2 / d\Phi$ at the beginning of the titration (i.e., $\beta_2^{1/2}[\mathrm{X}] = 0$) correspond to

$$\left.\frac{d\alpha_1}{d\Phi}\right|_{\Phi=0} = \frac{M_T\beta_1}{1+M_T\beta_1} = \frac{c_0}{1+c_0} \quad \text{(S86a)}$$

$$\left.\frac{d\alpha_2}{d\Phi}\right|_{\Phi=0} = 0 \quad \text{(S86b)}$$

where $c_0 = M_T \cdot \beta_1$. Similarly, the values of the differentials $d\alpha_1 / d\Phi$ and $d\alpha_2 / d\Phi$ at 50% binding saturation (i.e., $\beta_2^{1/2}[\mathrm{X}] = 1$) correspond to

$$\left.\frac{d\alpha_1}{d\Phi}\right|_{\Phi^{50\%}} = 0 \quad \text{(S87a)}$$

$$\begin{aligned}\left.\frac{d\alpha_2}{d\Phi}\right|_{\Phi^{50\%}} &= \frac{M_T(\beta_1 + 2\beta_2^{1/2})}{(2+\beta_1/\beta_2^{1/2})^2 + 2M_T(\beta_1 + 2\beta_2^{1/2})} \\ &= \frac{c_0 + 2c_{1/2}}{4\cdot(1+\rho^{-1/2})^2 + 2\cdot(c_0 + 2c_{1/2})}\end{aligned} \quad \text{(S87b)}$$

where $c_{1/2}$ was defined in eq S80, and ρ is the stepwise cooperativity parameter (eq 28). The previous differentials can be used to independently evaluate the values of ΔH_1 and ΔH_2. For example, ΔH_1 can be evaluated with the expression

$$dH_{\Phi\to 0} = \frac{\Delta H_1 \cdot c_0}{1+c_0} \tag{S88}$$

since the contribution of $d\alpha_2 / d\Phi$ in the limit of $\Phi \to 0$ is nearly zero. Similarly, ΔH_2 can be evaluated with the expression

$$dH_{\Phi^{50\%}} = \frac{\Delta H_2 \cdot (c_0 + 2c_{1/2})}{4 \cdot (1+\rho^{-1/2})^2 + 2 \cdot (c_0 + 2c_{1/2})} \tag{S89}$$

since the heat per mole contribution of $d\alpha_1 / d\Phi$ at $\Phi_{50\%}$ is also nearly zero.

6. *Numerical Evaluation of the DBM*

Similar to section B.3, the analysis of an ITC experiment with the DBM for a receptor with n = 2 requires an expression to calculate the free ligand concentration [X] as a function of the volume injected. First, we use the expressions for X_T, M_T and Φ (eqs S49a-S49c) to correct the concentration of the binding species due to the dilution and overflow of the titration cell. Then, we derive an explicit expression for the mass balance equation with the form

$$[\mathrm{X}]^3 + p[\mathrm{X}]^2 + q[\mathrm{X}] + r = 0 \tag{S90a}$$

where

$$\begin{aligned} p &= \left(\frac{\beta_1}{\beta_2} + M_T(2-\Phi)\right) \\ q &= \frac{1}{\beta_2}\left(1 + M_T\beta_1(1-\Phi)\right) \\ r &= -\frac{M_T\Phi}{\beta_2} \end{aligned} \tag{S91b}$$

The real root of the polynomial is given by

$$[\mathrm{X}] = \frac{-p + 2(p^2 - 3q)^{1/2}\cos(\theta/3)}{3} \tag{S92a}$$

$$\theta = \arccos\left(\frac{-2p^3 + 9pq - 27r}{2(p^2 - 3q)^{3/2}}\right) \tag{S92b}$$

which is similar to the expressions derived by Wang[11] for the analysis of a receptor with competitive binding interactions for two ligand species. The exact algebraic solution of [X] (Eq S92) is valid for receptors with either negative or non-cooperative binding interactions (i.e., $\beta_1^2 \leq 4\ \beta_2$). Receptors with positive cooperative interactions cannot be analyzed with eq S92. Thus, approximate methods such as the Secant[6] and Newton's method[12] are a more general approach to calculate the real root of the mass-balance polynomial. We use the FindRoots command in IGOR Pro (WaveMetrics Inc., Lake Oswego, OR, USA), which uses the Jenkins-Traub algorithm,[13] to calculate the concentration of free ligand from eq 17d (main text). Finally, we use the value of [X] to evaluate the appropriate DBM for a receptor with n = 2 and the binding parameters are optimized using a non-linear regression routine. For the preliminary analyses, we prefer eq S59, where the DBM is expressed in terms of the cumulative binding constants β_i and the cumulative enthalpies of binding ΔH_i, and which also includes the terms N and Δh_{dil}.

D. Comparison of the DBMs with the Built-In Models in Origin.

Table S1 shows the fitting results of the non-linear regression analysis with the general binding model and a DBM that accounts for cooperative binding interactions between the two sites. Interestingly, the value of χ_ν^2 = 1.92 is the same for all the DBMs analyzed (see Table 2, main text and Table S2). This implies that the all the DBMs are mathematically equivalent. The major difference between the different DBMs, besides the binding parameters obtained, is the correlation matrix for each model. A large correlation value (e.g. -1.0 or 1.0) also shows that the binding parameters are multiplication factors in the explicit expression of the DBM. For example, we observe a large correlation between the cumulative binding constants β_1 and β_2 (Corr(β_1, β_2) = 0.99) since the parameters β_2 and β_1 are multiplied eq S58. However, the binding constants k_{11} and k_{21} in Table S2 show almost no correlation (Corr(k_{11}, k_{21})= 0.14), since these binding parameters are added in eqs S74a-S74e.

The non-linear regression analyses of the averaged *dH* titration curves performed in Origin with the *sequential-sites* and the *two-sites* built-in models are shown in Figure S5. In comparison

Table S1. Thermodynamic and statistical parameters for the titration of Gd(III) with sodium citrate in MES Buffer (100 mM, pH 5.5) obtained with the general DBM and a DBM constrained for cooperative binding sites.

	General Binding Model[a]							
Binding Parameters	**Best Fit Value**	**+/-**	**Correlation Matrix**					
N	1.01	0.001	1	0.24	0.30	-0.13	0.89	-0.26
$\log \beta_1$ (−log M)	7.07	0.03		1	0.99	-0.38	0.22	-0.13
$\log \beta_2$ (−2·log M)	11.21	0.03			1	-0.38	0.31	-0.22
ΔH_1 (kJ mol^{-1})	-1.84	0.01				1.00	0.02	0.04
ΔH_2 (kJ mol^{-1})	-18.41	0.06					1.00	-0.58
Δh_{dil} (kJ mol^{-1})	0.19	0.01						1.00
χ^2_ν	**1.92**							
	Cooperative Binding Model							
Binding Parameters	**Best Fit Value**	**+/-**	**Correlation Matrix**					
N	1.01	0.001	1	0.24	-0.18	-0.13	0.89	-0.26
$\log k$ (−log M)	6.77	0.03		1	-0.99	-0.38	0.33	-0.13
$\log \kappa$	-2.34	0.03			1	0.38	-0.24	0.04
Δh (kJ mol^{-1})	-1.84	0.01				1	-0.28	0.04
$\Delta \eta$(kJ mol^{-1})	-14.73	0.07					1	-0.57
Δh_{dil} (kJ mol^{-1})	0.19	0.01						1
χ^2_ν	**1.92**							

a. The general DMB in terms of the cumulative binding parameters for a receptor with $n = 2$ is shown in eq S59.

with our DBM constrained for sequential binding interactions (eq S67), the built-in model in Origin (Figure S5A) does not contain an adjustable parameter to correct for small errors in the concentration of the binding species (e.g. N, where $M_T^* = N \cdot M_T$) or for the enthalpy of dilution for the free ligand species (Δh_{dil}). In Origin, we adjust the heat of dilution manually by adding or subtracting small heat values (ca. 0.02 kJ·mol^{-1}) from the experimental data, which shifts the titration curve vertically. This process is repeated until we obtain a minimum value for the reduced chi-square parameter (χ^2_ν). Although the concentration of receptor could be adjusted in a similar way, we believe this is a time consuming process that would be better handled with a computer algorithm.

In the bottom panel of Figure S5A, we observe a large deviation between the experimental and the fitted values for titration points near Φ = 1.0. This large discrepancy is due to the absence of the fitting parameter *N*, which shifts the inflection point of the *dH* titration curve. As a result, we obtained a large value for the reduced chi-square parameter (χ_ν^2 = 5.45) with the sequential binding sites model. As shown in the section 5.4 of the main text, our DBM gives a much lower χ_ν^2 value by including the fitting parameters *N* and Δh_{dil} in the algorithm.

In Figure S5B, the titration curve was analyzed using the *two-sites* model included in Origin, which is based on the assumption of independent binding sites. This binding model contains two stoichiometry coefficients (N_1, N_2), one for each site, and it is similar to a DBM with independent interactions between the two sites (eq S75). The DBM for a receptor with two sites and two stoichiometry coefficients, as the one included in Origin, is represented with the binding polynomial

$$P_M = (1 + k_{11}[\mathrm{X}])^{N_1} (1 + k_{21}[\mathrm{X}])^{N_2} \tag{S93}$$

After following the steps described in section B.3, we obtain the following expression to evaluate the calorimetry titration curves

$$dH = \frac{\Delta h_1 c_{(1)}}{c_{(1)} + (P_{M(1)})^2 \left(1 + \dfrac{c_{(2)}}{(P_{M(2)})^2}\right)} + \frac{\Delta h_2 c_{(2)}}{c_{(2)} + (P_{M(2)})^2 \left(1 + \dfrac{c_{(1)}}{(P_{M(1)})^2}\right)} \tag{S94a}$$

where

$$c_{(1)} = N_1 M_T \cdot k_{11} \quad , \quad c_{(2)} = N_2 M_T \cdot k_{21}$$
$$P_{M(1)} = 1 + k_{11}[\mathrm{X}] \quad , and \quad P_{M(2)} = 1 + k_{21}[\mathrm{X}] \tag{S94b}$$

If the DBM contains a single stoichiometry parameter (*N*), the terms N_1 and N_2 in eqs S93 and S94 are equivalent (i.e., $N = N_1 = N_2$) and the dimensionless parameters $c_{(1)}$ and $c_{(2)}$ are evaluated with the expressions

$$c_{(1)} = N \cdot M_T \cdot k_{11} \quad , and \quad c_{(2)} = N \cdot M_T \cdot k_{21} \tag{S95}$$

Table S2. Thermodynamic and statistical parameters for the titration of Gd(III) with sodium citrate in MES Buffer (100 mM, pH 5.5) obtained with the DBM constrained for two independent binding sites.

Binding Parameters	**DBM**		**Origin**		**Correlation Matrix**					
	Best Fit Value	+/-	Best Fit Value	+/-						
N *or* N_1	1.01	0.001	0.97	0.003	1	0.24	0.53	-0.15	0.90	-0.26
N_2			1.02	0.004						
log k_{11} (−log M)	7.07	0.03	7.19	0.10		1	0.14	-0.48	0.30	-0.13
log k_{21} (−log M)	4.13	0.004	4.14	0.01			1	-0.05	0.76	-0.78
Δh_1 (kJ mol^{-1})	-1.83	0.01	-2.03	0.01				1	-0.16	0.05
Δh_2 (kJ mol^{-1})	-16.59	0.07	-16.72	0.07					1	-0.58
Δh_{dil} (kJ mol^{-1})	0.19	0.01	0.21[a]							1
χ_v^2	**1.92**[b]		**1.96**							

a. In Origin, the heat of dilution was adjusted manually by adding or subtracting small heat values (ca. 0.02 kJ·mol^{-1}) until we obtain a minimum value for χ_v^2.

b. We also performed non-linear regression analysis with the DBM where the heat of dilution Δh_{dil} was held constant. This analysis gave a similar χ_v^2 value of 1.91.

As in the case of data fitted to the sequential sites model in Origin, the heat of dilution was adjusted manually until we obtained a minimum value of χ_v^2. The residuals obtained with the *two-sites* model show a random distribution of the difference between the experimental and the fitting points (bottom panel, Figure S5B). Thus, this model would seem as a better choice for the experimental data in comparison to the sequential sites model included in Origin. However, as described below, the *two-sites* model is over-parameterized since only one stoichiometry coefficient is required.

We summarize the fitting results of the non-linear regression analysis with the binding models that account for independent binding sites in Table S2. There is good agreement between the fitting values obtained with the DBM constrained for two independent sites with one stoichiometry coefficient and the model for *two-sites* in Origin. As noted in the main text, one would expect a lower χ_v^2 with a nested model that contains an additional stoichiometry parameter. However, the χ_v^2 values for the DBM and the *two-sites* model in Origin are nearly equal. Both models contain the same number of fitting parameters; hence, it is not possible to

directly perform an *F*-test[14] to evaluate the validity of the additional stoichiometry parameter. In order to make a meaningful comparison with the F-test, we performed an additional non-linear regression analysis of the titration where the heat of dilution in the DBM was held constant. The resulting reduced chi-square value for this regression analysis was $\chi^2_\nu = 1.91$, which gives an $F_\chi = 0.67$ and $F_{\mathrm{Crit},0.05} = 4.03$ for a model with an additional binding parameter. Since $F_\chi < F_{\mathrm{Crit}}$, there is at least 95% probability that the additional binding parameter is not statistically valid.

E. References

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F. Figures

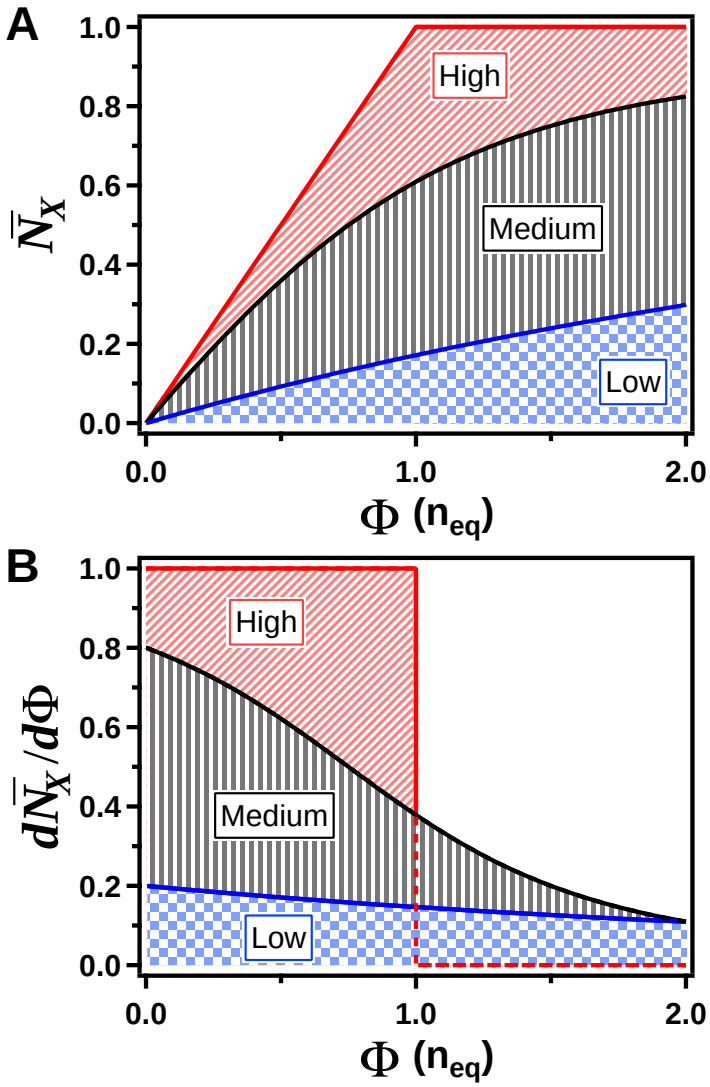

Figure S1. Titration representation of the average ligand binding $\bar{N}_X$ and differential binding satuation $d\bar{N}_X / d\Phi$ for a receptor with *one* binding site (n = 1) for a titration range $0 \geq \Phi \geq 2.0$ with three ranges of c-values defined as follows: $c \geq 4$ is considered high, gives initial values for $d\bar{N}_X / d\Phi \geq 0.8$, and is represented by (▨); c in the range 4> c > 0.25 is considered medium gives initial values for $d\bar{N}_X / d\Phi$ in the range $0.8 > d\bar{N}_X / d\Phi > 0.2$, and is represented by(▥); and $c \leq 0.5$ is considered low, gives initial values for $d\bar{N}_X / d\Phi \leq 0.2$, and is represented by (▩).

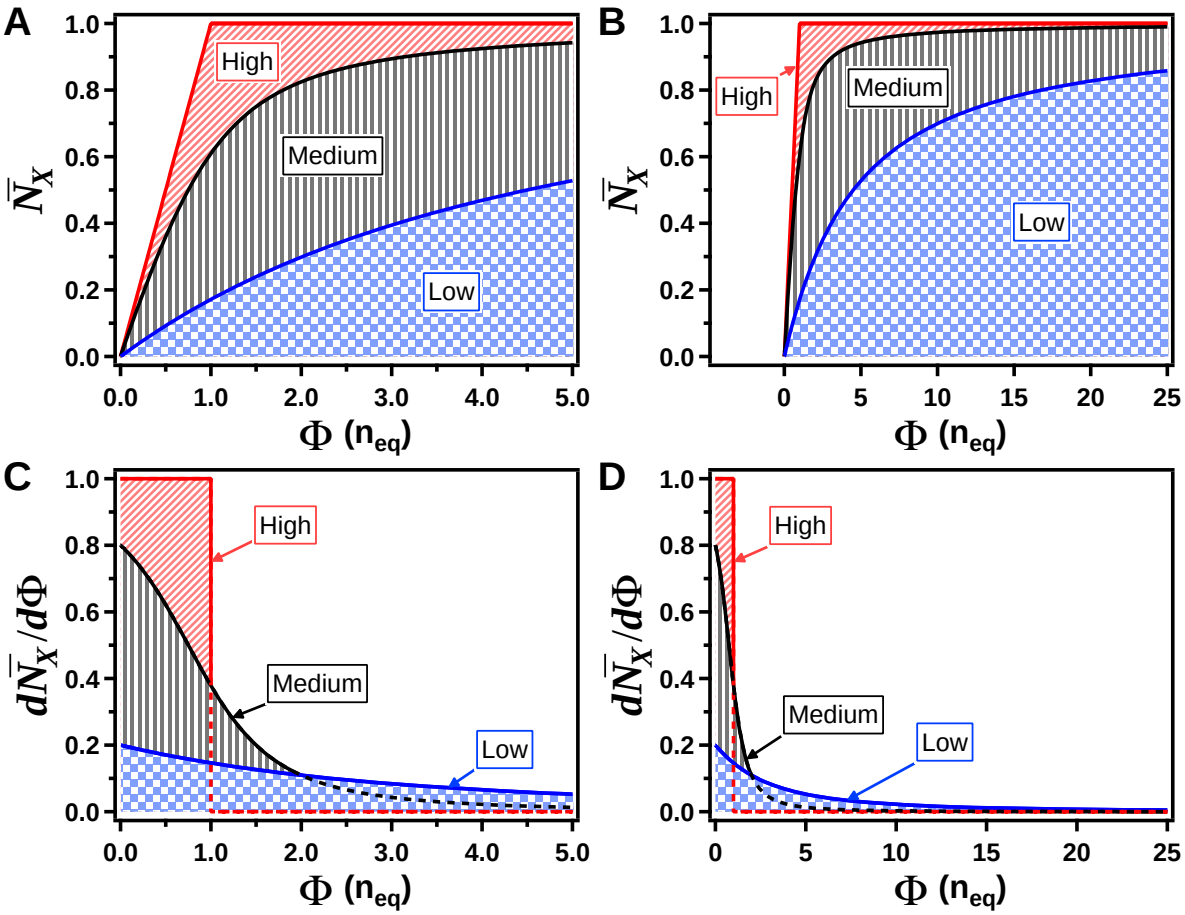

Figure S2. Titration representation of $\bar{N}_X$ and $d\bar{N}_X / d\Phi$ for a receptor with *one* binding site (n = 1) and three ranges of c-values defined as follows: $c \geq 4$ is considered high, gives initial values for $d\bar{N}_X / d\Phi \geq$ 0.8, and is represented by ( ); c in the range $4 > c > 0.25$ is considered medium, gives initial values for $d\bar{N}_X / d\Phi$ in the range $0.8 > d\bar{N}_X / d\Phi > 0.2$, and is represented by( ); and $c \leq 0.25$ is considered low, gives initial values for $d\bar{N}_X / d\Phi \leq 0.2$, and represented by ( ). By increasing the end value of the degree of titration from 2.0 to 5.0 (A) and from 2.0 to 25.0 (B), we can the observe the hyperbolic shape of $\bar{N}_X$ for a titration with a medium and low c value, respectively. C) The differential binding curve $d\bar{N}_X / d\Phi$ has a nearly sigmoidal shape for a titration with medium c values in the titration range from $\Phi = 0$ to $\Phi = 5.0$. C) By increasing the end value of the degree of titration from 2 to 25, we observe the hyperbolic shape of a titration with low c values. D) The plot of $d\bar{N}_X / d\Phi$ over the titration range from $\Phi = 0$ to $\Phi = 25$ shows that a titration with either a medium or a low c value appears to have a decaying exponential shape. The values of differential binding seem to be clustered in the degree of titration $\Phi < 10$. The value of $d\bar{N}_X / d\Phi$ changes at a slow rate for titration points $\Phi > 10$.

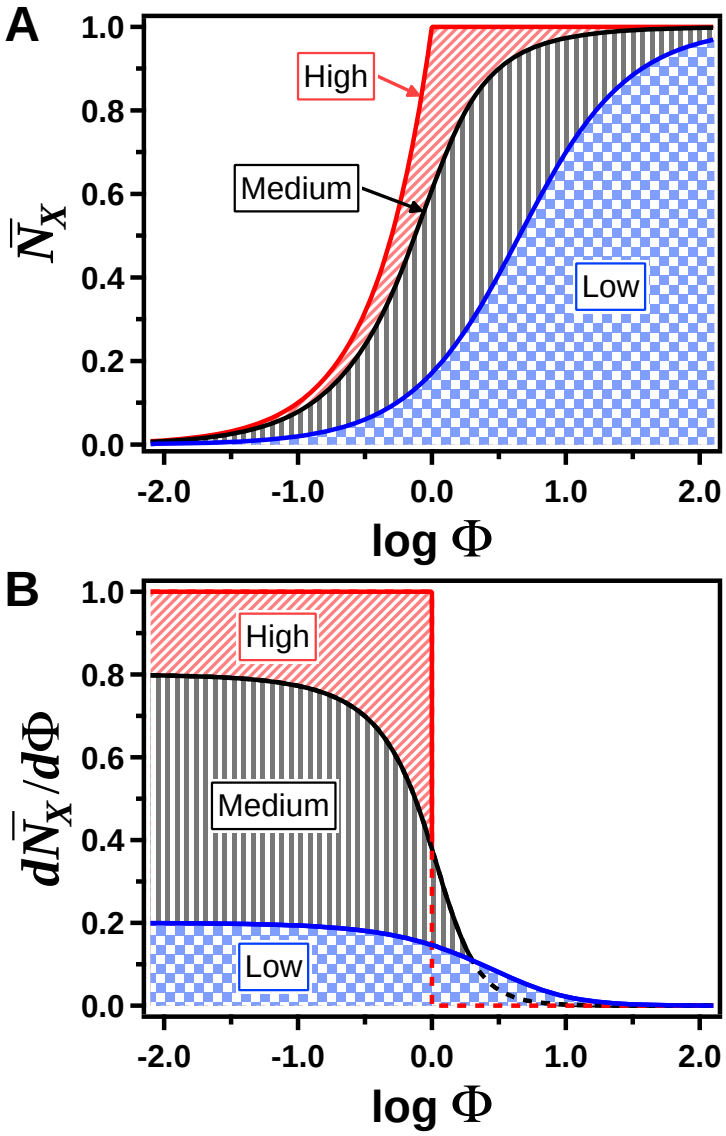

Figure S3. Semi-logarithmic representation of $\bar{N}_X$ and $d\bar{N}_X / d\Phi$ for a receptor with *one* binding site (n = 1) and three ranges of c-values defined as follows: $c \geq 4$ is considered high and gives initial values $d\bar{N}_X / d\Phi \geq 0.8$. and is represented by (▨); c in the range $4 > c > 0.25$ is medium and is represented by(▥); and $c \leq 0.25$ is low and is represented by (▩).

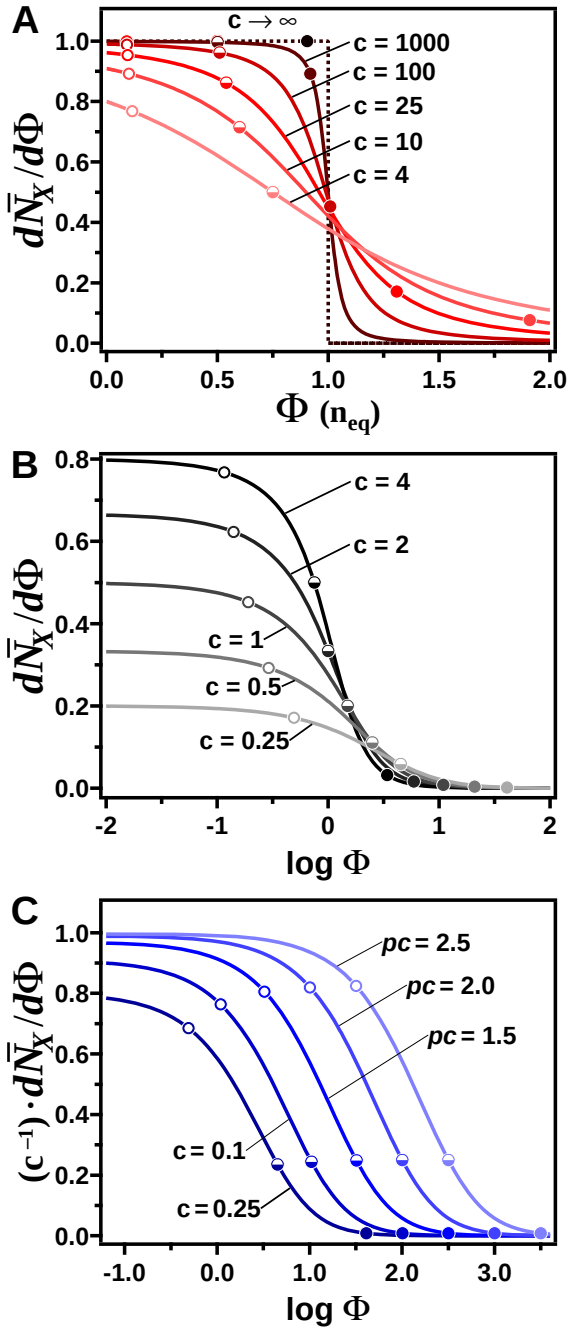

Figure S4. Titration representation of differential binding curves $d\bar{N}_X / d\Phi$ for a receptor with *one* binding site (n = 1) in each titration range: A) Differential binding curves with a high c value ($c \geq 4$) have a sigmoidal shape when plotted with respect to the degree of titration Φ. B) The sigmoidal shape of $d\bar{N}_X / d\Phi$ for titrations with medium c values ($4 > c > 0.25$) is shown by using a semi-logarithmic representation of the degree of titration (i.e., log Φ). C) Titration experiments with a weak c value ($c \leq 0.25$) also have a sigmoidal shape when plotted with respect to log Φ and the differential binding curve is divided by c. The term pc is the negative log base 10 for c as given by $pc = -\log c$.

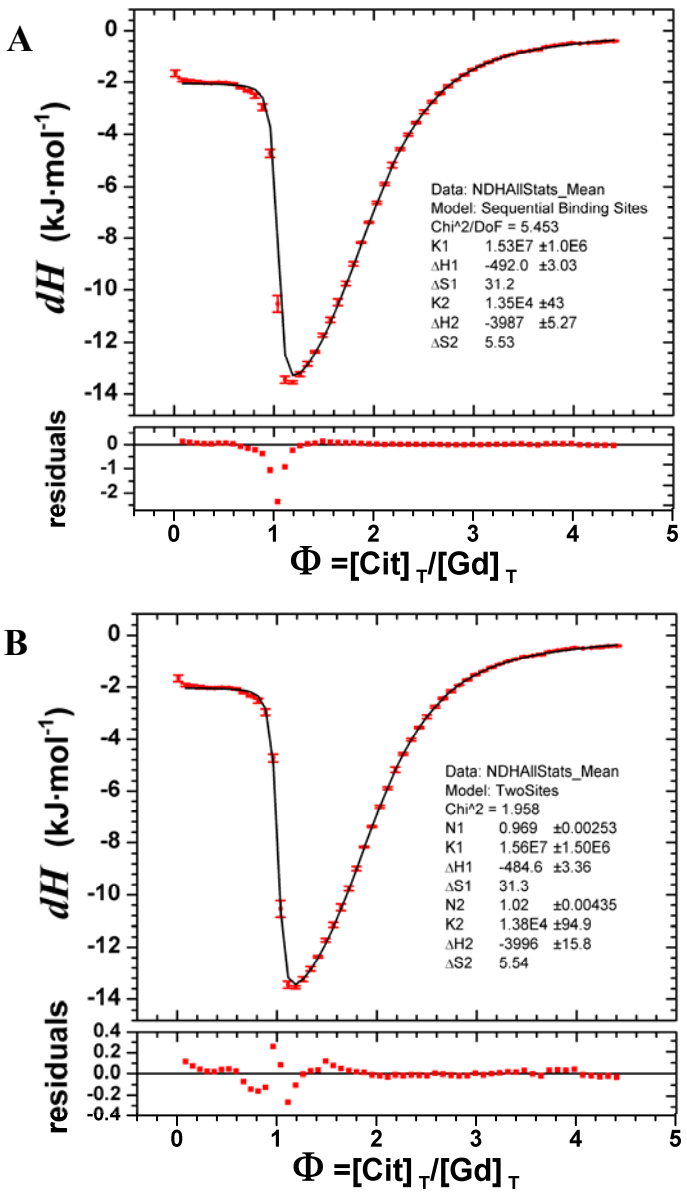

Figure S5. Titration curves for the averaged the differential heat per mole values from three titrations. The error bars in each titration curve indicate the standard deviation (σ_k) from the averaged *dH* values. The solid lines indicate the fitting curves obtained with the *sequential binding sites* (A) and the *two-sites* (B) models in Origin 7.0. The residuals obtained from the weighted non-linear least-squares regression analysis for each model are shown at the bottom of each panel. The reduced chi-square values (χ_v^2) for the sequential and independent bindings sites are 5.45 and 1.96, respectively.