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Short communication

Biphasic thermodynamic characterization of interactions by quantitative affinity chromatography and biosensor technology

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Abstract

An alternative theory of quantitative affinity chromatography is developed to take into account the fact that interaction between the soluble ligate and immobilized affinity sites is confined to the stationary phase. The affinity constant deduced from the previous single-phase theory reflects the product of the partition coefficient and the equilibrium constant defined in terms of concentrations prevailing in the stationary phase; and the former total concentration of affinity sites also includes an extra term to take into account the fractional volume occupied by stationary phase. Although corresponding quantitative relationships have been developed for the characterization of interactions by biosensor technology, it is recommended that the current single-phase analysis be retained to allow comparisons of affinity constants with those deduced by the traditional kinetic analysis. © 1998 Elsevier Science B.V.

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1. Introduction

Since the inception of quantitative affinity chromatography [1–3] many characterizations of interactions by this technique have been based on a single-phase thermodynamic model. Such consideration of immobilized affinity sites on the chromatographic matrix to be distributed uniformly throughout a single phase can be justified thermodynamically on the grounds of their potential for access by all partitioning solute molecules (A) throughout the volume accessible to A [3]. However, that thermodynamically acceptable model is unrealistic physically inasmuch as interactions with affinity sites must occur within the confines of the stationary-phase fraction of the total volume. Although various attempts have been made to incorporate the existence of the second phase into kinetic analyses of affinity chromatographic data (see, e.g., [4–8]), the advent of biosensor-based characterization of biphasic affinity systems [9–11] has rekindled interest in the problem. The aim of the present communication is to develop the basic quantitative expressions for thermodynamic characterization of affinity chromatographic interactions in terms of a biphasic system; and thereby to establish the inter-relationships between parameters so determined and their counterparts derived on the basis of the single-phase model.

2. Theory

Consider a situation in which the stationary (gel)

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phase comprises a fraction ϕ of the total volume V. Affinity sites X, present at a total concentration $[\bar{X}]_s$, are clearly confined to the stationary phase volume, ϕV, whereas the partitioning solute (ligate, A) is distributed throughout the liquid and stationary phases. The concentration of free ligate within the gel phase, $[A]_s$, is related to that in the liquid phase, [A], by the expression

$$[A]_s = \sigma_A[A] \quad (1)$$

where σ_A is the partition coefficient [12,13]. Because interaction between ligate and affinity sites is necessarily confined to the stationary phase, an appropriate definition of the concentration of bound ligate is in terms of concentrations pertaining to that phase. For a ligate that is univalent in its interaction with equivalent and independent affinity sites (X), the rectangular hyperbolic dependence of the concentration of complexed ligate upon that of free ligate is therefore written

$$[\bar{A}]_s - [A]_s = (K_{AX})_s[\bar{X}]_s[A]_s/[1 + (K_{AX})_s[A]_s] \quad (2)$$

where $[\bar{A}]_s$ denotes the total ligate concentration within the stationary phase, and $(K_{AX})_s$ is the intrinsic binding constant for the interaction when concentrations are expressed in this manner. We now need to convert Eq. (2) into an expression in terms of more readily determinable experimental variables. There are several situations to consider.

2.1. Partition equilibrium measurements

In partition equilibrium experiments the ligate concentrations available to the experimenter are the total concentration $\left[\bar{\bar{A}}\right]$ (molar amount divided by V) and the free concentration in the liquid phase, [A]. From considerations of mass conservation it follows that

$$\left[\bar{\bar{A}}\right]V = [A](1 - \phi)V + [\bar{A}]_s\phi V \quad (3a)$$

whereupon

$$[\bar{A}]_s = \left\{\left[\bar{\bar{A}}\right] - [A](1 - \phi)\right\}/\phi \quad (3b)$$

Substitution of Eqs. (1) and (3b) into the binding expression (Eq. (2)) then yields

$$\left\{\left[\bar{\bar{A}}\right] - [A]\{1 - \phi(1 - \sigma_A)\}\right\}/\phi = \sigma_A(K_{AX})_s[\bar{X}]_s[A]/\{1 + \sigma_A(K_{AX})_s[A]\} \quad (4)$$

Although precise characterization of the interaction in terms of this model requires knowledge of both ϕ and σ_A, experiments are frequently performed under conditions where $\sigma_A \to 1$ and $\phi \ll 1$ [14,15]. Under those conditions Eq. (4) simplifies to

$$\left(\left[\bar{\bar{A}}\right] - [A]\right)/\phi \approx \sigma_A(K_{AX})_s[\bar{X}]_s[A]/\{1 + \sigma_A(K_{AX})_s[A]\} \quad (5)$$

Irrespective of whether the complete expression or its approximate form is used, the measurement of [A] for a series of $\left[\bar{\bar{A}}\right]$ affords a means of evaluating $[\bar{X}]_s$ and $(K_{AX})_s$ provided that values of ϕ and σ_A are available.

2.2. Recycling partition chromatography

Recycling partition chromatography [14,15] is a variant of the simple partition protocol which allows the collection of binding data in the form of a stepwise titration. The fact that successive additions of concentrated ligate solution are made to the same slurry of affinity matrix ensures that the volume of the gel phase remains constant, whereupon changes in ϕ reflect solely the systematic increase in V with each ligate addition. On the basis of the substitution $\phi = (V_i/V)\phi_i$, where ϕ_i is the initial fractional gel volume in the system with initial volume V_i, Eq. (5) may be written in the form

$$(V/V_i)\left(\left[\bar{\bar{A}}\right] - [A]\right) = \sigma_A\phi_i(K_{AX})_s[\bar{X}]_s[A]/\{1 + \sigma_A(K_{AX})_s[A]\} \quad (6)$$

Thus, even in instances where neither ϕ_i nor σ_A is of known magnitude, nonlinear regression analysis of $(V/V_i)(\left[\bar{\bar{A}}\right] - [A])$ in terms of a rectangular hyperbolic dependence upon [A] (Eq. (6)) yields estimates of the two products $\sigma_A(K_{AX})_s$ and $\phi_i[\bar{X}]_s$.

2.3. Frontal affinity chromatography

In frontal chromatography [16,17] sufficient ligate

solution is applied to the column to generate an elution profile that contains a plateau region with the ligate concentration equal to that applied. Although such action ensures knowledge of [A] as the applied concentration, there remains the problem of deducing the magnitude of $\left[\bar{\bar{A}}\right]$ to which the elution volume, $\bar{V}_A$, refers. On the grounds that the product $\bar{V}_A[A]$ defines the amount of ligate on an affinity column with bed volume V [18,19], we may write

$$\bar{V}_A[A] = (1-\phi)V[A] + (\phi V)\sigma_A[A] + (\phi V)\{[\bar{A}]_s - [A]_s\} \quad (7)$$

as the statement of mass conservation. Setting the final term on the right-hand side of Eq. (7) equal to zero leads to the expression

$$V_A^* = V[1-\phi(1-\sigma_A)] \quad (8)$$

for the relationship between basic column chromatographic characteristics (V, ϕ, σ_A) and V_A^*, the consequent elution volume of A in the absence of chemical interaction with the affinity matrix: V_A^* may be determined by, for example, conducting an experiment on A in the presence of a saturating concentration of a competitive inhibitor of the ligate–matrix interaction. Combination of Eqs. (7) and (8) gives

$$\{[\bar{A}]_s - [A]_s\} = [A](\bar{V}_A - V_A^*)/(\phi V) \quad (9)$$

whereupon the chromatographic counterpart of Eq. (2) becomes

$$(\bar{V}_A - V_A^*)/V = \phi\sigma_A(K_{AX})_s[\bar{X}]_s/\{1+\sigma_A(K_{AX})_s[A]\} \quad (10)$$

The two parameters that dictate the rectangular hyperbolic dependence of $(\bar{V}_A - V_A^*)/V$ upon loading ligate concentration [A] are thus the products $\phi[\bar{X}]_s$ and $\sigma_A(K_{AX})_s$.

2.4. Biosensor-based measurement of partition equilibrium

Whereas the methods considered thus far have relied upon indirect determination of the concentration of bound ligate, $[\bar{A}]_s - [A]_s$, from the concentration of free ligate remaining in the liquid phase, the advent of biosensor technology [20,21] has rendered possible the direct monitoring of complexed ligate. From the thermodynamic viewpoint the collection of partition equilibrium data by BIAcore technology is equivalent to frontal chromatography in the sense that ligate solution flows across the affinity matrix attached to the sensor surface until equilibrium is achieved, thereby establishing [A] as the ligate concentration injected into the instrument. On the other hand, because of its cuvette-based design, the IAsys instrument yields data more akin to those obtained by the partition equilibrium method. The appearance of the progress curve for complex formation is qualitatively similar for both instruments.

On the basis of direct proportionality between equilibrium response, R_e, and the concentration of complexed ligate, the counterpart of Eq. (2) for the BIAcore instrument may be written

$$R_e = F_B\{[\bar{A}]_s - [A]_s\} = F_B[\bar{X}]_s\sigma_A(K_{AX})_s[A]/\{1+\sigma_A(K_{AX})_s[A]\} \quad (11)$$

Nonlinear regression analysis of (R_e, [A]) data in terms of a rectangular hyperbolic dependence of R_e upon [A] thus yields values of $\sigma_A(K_{AX})_s$ and $F_B[\bar{X}]_s$, where F_B is the proportionality constant between response and bound concentration for the BIAcore instrument.

The concentration of bound ligate is also monitored in the IAsys instrument; but, in a manner reminiscent of that adopted for the static and recycling partition equilibrium methods, a mass conservation argument must be used to deduce the magnitude of [A] from the initial amount of ligate introduced into the cuvette, $V\left[\bar{\bar{A}}\right]$, and the amount of complexed ligate. Thus,

$$V\left[\bar{\bar{A}}\right] = \{(1-\phi)V\}[A] + (\phi V)\sigma_A[A] + (\phi V)(R_e/F_I) \quad (12)$$

where F_I is the proportionality constant between response and bound ligate concentration for the IAsys instrument. This expression is clearly the counterpart of Eqs. (3a) and (3b) for partition equilibrium experiments; but on this occasion it is used for the evaluation of [A]. Specifically,

$$[A] = \left\{\left[\bar{\bar{A}}\right] - \phi(R_e/F_I)\right\}/[1-\phi(1-\sigma_A)] \quad (13)$$

The fact that the magnitude of ϕ is small (see later) sometimes leads to acceptability of the approximation $[A] \approx \left[\bar{\bar{A}}\right]$, whereupon Eq. (11) with F_I and $\left[\bar{\bar{A}}\right]$ substituted for F_B and [A], respectively, may be used for the quantitative characterization of an interaction by IAsys biosensor technology. For many systems, however, the invalidity of that approximation [22] renders necessary the use of Eq. (13) to evaluate [A]. Thus the general IAsys counterpart of Eq. (11) becomes

$$R_e = F_I[\bar{X}]_s \sigma_A (K_{AX})_s \left\{ \left[\bar{\bar{A}}\right] - \phi(R_e/F_I) \right\} / \{1 - \phi(1 - \sigma_A) + \sigma_A (K_{AX})_s\} \left[\left[\bar{\bar{A}}\right] - \phi(R_e/F_I) \right] \quad (14)$$

Inasmuch as the design of the IAsys instrument is conducive to the conduct of stepwise titrations [22], the volume fraction of gel phase may again be expressed as $(V_i/V)\phi_i$. Irrespective of the manner in which magnitudes are assigned to [A], the important conclusion to emanate from the above considerations is that the rectangular hyperbolic dependence of R_e upon free ligate concentration yields $F_I[\bar{X}]_s$ and $\sigma_A(K_{AX})_s$ as the evaluated parameters.

3. Consideration of experimental results

The preceding biphasic theory of quantitative affinity chromatography was developed to provide a thermodynamic description with greater physical relevance than its single-phase predecessor [3]. It is therefore appropriate to reconsider results from published partition equilibrium, frontal chromatographic and biosensor studies in order to assess the consequences of the physical shortcomings of the earlier thermodynamic model. Those deliberations are commenced by reappraising results from a recycling partition equilibrium study of the biospecific interaction between antithrombin and heparin-Sepharose.

3.1. Recycling partition equilibrium studies

This recycling partition study of the interaction of antithrombin with heparin-Sepharose [15] entailed successive additions of ligate aliquots to a stirred slurry (approx. 6 ml) of affinity matrix (10 mg) comprising heparin covalently attached to Sepharose-6B. For this system with a gel-phase volume in the vicinity of 0.04 ml, the value of ϕ is about 0.0067. Furthermore, on the grounds that the molecular mass fractionation range of Sepharose-6B is 10 000–$4 \cdot 10^6$, the partition coefficient of antithrombin (M_A = 58 000) is likely to differ little from unity. Inasmuch as the assumptions inherent in the simplification of the general partition equilibrium expression (Eq. (4)) to its approximate form (Eq. (5)) are thus fulfilled, Eq. (6) becomes the appropriate expression for analysis of the recycling partition data. The parameters $\phi[\bar{X}]_s$ and $\sigma_A(K_{AX})_s$ are thus to be evaluated from the rectangular hyperbolic dependence of $(V/V_i)(\left[\bar{\bar{A}}\right] - [A])$ upon [A].

Reference to Fig. 4 of Ref. [15] shows that this dependence has already been used to analyze the results for the antithrombin–heparin-Sepharose system in terms of the single-phase theory of quantitative affinity chromatography, the relevant expression being

$$(V/V_i)\left(\left[\bar{\bar{A}}\right] - [A] \right) = K_{AX} \left[\bar{\bar{X}}\right]_i [A]/(1 + K_{AX}[A]) \quad (15)$$

Comparison of Eq. (15) with Eq. (6) shows that the capacity parameter described as $\left[\bar{\bar{X}}\right]_i$ in the single-phase theory is equivalent to the product $\phi_i[X]_s$ in the current analysis. Similarly, the operational affinity constant, K_{AX}, that is derived on the basis of single-phase theory, becomes $\sigma_A(K_{AX})_s$ when the thermodynamic argument is developed in terms of a biphasic system. As noted above, the partition coefficient is likely to be only slightly smaller than unity, whereupon there should be little difference between the magnitudes of K_{AX} and $(K_{AX})_s$, the binding constant defined on the basis of concentrations in the gel phase. On the other hand, a much greater disparity resides in the values assigned to the total concentration of matrix sites. On the basis that ϕ_i would have been in the vicinity of 0.0067 (i.e., 6/0.04), the total concentration of affinity sites within the gel matrix becomes 0.33 mM (cf. 2.2 μM in the single-phase analysis). Nevertheless, both analyses predict a common gel capacity, $V_i\left[\bar{\bar{X}}\right]_i$ and $\phi_i V_i[\bar{X}]_s$.

This development of the biphasic treatment has been a response to criticism that the inclusion of the (V/V_i) term in Eq. (15) to account for a systematic decrease in $\left[\bar{\bar{X}}\right]$ with increasing volume V of the system must be invalid: confinement of affinity sites to the gel phase must surely signify that their concentration is insensitive to the aqueous and hence total volume. Adoption of that physically correct stance has still led to incorporation of the same volume correction factor, which then reflects the ever-decreasing volume fraction of the gel phase (ϕ). The important point to note is the equivalence of the two treatments – a necessary outcome, of course, because of the nature of thermodynamics.

3.2. Reappraisal of frontal affinity chromatographic studies

The previous section on partition equilibrium studies has already made the point that thermodynamic analyses based on consideration of systems in terms of single-phase and biphasic theory must be equivalent. That inference must clearly pertain to all forms of quantitative affinity chromatographic investigation. In this reconsideration of results obtained by frontal affinity chromatography we again introduce the expression for thermodynamic characterization in terms of single-phase theory, namely,

$$(\bar{V}_A - V_A^*)/V = K_{AX}\left[\bar{\bar{X}}\right][A]/(1 + K_{AX}[A]) \qquad (16)$$

Comparison of Eq. (16) with Eq. (10), its counterpart in biphasic theory, reveals the expected substitution of $\left[\bar{\bar{X}}\right]$ for $\phi[\bar{X}]_s$ and of K_{AX} for $\sigma_A(K_{AX})_s$. Specific attention is accorded the biospecific interaction of cytochalasin B with erythrocyte membranes embedded within the beads of a 0.8-ml Superdex column [17] – a system for which the major discrepancy between parameter estimates is in the binding constant rather than the concentration of affinity sites.

From a plot of the dependence of $(\bar{V}_A - V_A^*)/V$ upon cytochalasin B concentration according to the reciprocal transform of Eq. (16), estimates of $1.0 \cdot 10^7\ M^{-1}$ and 1.1 μM were obtained for K_{AX} and $\left[\bar{\bar{X}}\right]$, respectively [17]. However, this notion of seemingly strong interaction is tempered somewhat by consideration of the affinity constant parameter as the product $\sigma_A(K_{AX})_s$ according to biphasic theory. From Figs. 7B and 7C of the original publication [17], $V_A^* = 8.5$ ml for a column with a bed volume of 0.8 ml. On the grounds that $\phi \approx 0.7$ for a packed column of spherical beads, Eq. (8) yields a value of 14.8 for the partition coefficient (σ_A). Inasmuch as the cytochalasin B concentration within the Superdex beads is thus almost 15-fold greater than [A], the binding constant in those terms, $(K_{AX})_s$, decreases to $8.1 \cdot 10^5\ M^{-1}$. On the other hand, because the fractional stationary phase volume (ϕ) is about 0.7, the total concentration of affinity sites increases by only 0.4 μM (1.2→1.6 μM) on adoption of the biphasic theory.

The important point to appreciate is that either thermodynamic description characterizes the chromatographic data correctly in terms of the model used for their analysis. Consequently, although the choice of model used to characterize the interaction is immaterial from the thermodynamic viewpoint, considerable caution needs to be exercised in assigning physical significance to the magnitudes of the binding constant and affinity-site concentration so determined.

3.3. Biosensor studies of ligate binding

The simpler and less equivocal thermodynamic model to apply to biosensor data is the original single-phase version because it obviates the necessity of specifying values for either ϕ or σ_A. Examples of such thermodynamic characterization include a study of the interaction between immobilized interleukin-6 and the soluble form of its biospecific receptor by BIAcore technology [23]; and one of the immunospecific interaction between carboxypeptidase A and an elicited monoclonal antibody immobilized on the sensor surface of an IAsys cuvette [22]. The reported affinity constants are therefore K_{AX}, i.e., the product of σ_A and $(K_{AX})_s$; and any attempt to evaluate $(K_{AX})_s$ is clearly predicated on satisfactory delineation of the magnitude of the partition coefficient σ_A. In that regard, Schuck [11] has employed a random-fibre model [24] of the carboxymethyldextran gel to derive the expression $\sigma_A = \exp(-0.000638\ M_A^{2/3})$ for the dependence of the partition coefficient upon molecular mass of a spherical ligate – a relationship

that predicts partition coefficients of 0.4 and 0.5 for the interleukin-6 receptor and carboxypeptidase A, respectively. In the absence of experimental methods for verifying the magnitude of σ_A, there is certainly merit in retaining K_{AX} and accepting the fact that it may underestimate the affinity constant within the gel phase by a factor of two or three.

There are also problems defining $[\bar{X}]_s$ in biosensor studies, because the thickness of the gel layer has only been defined to the extent that it should be less than 200 nm [25]: a value of 100 nm has been a popular estimate for theoretical purposes [11,26]. Combination of that compromise value with an area of 1.1 mm^2 for the microchannel base of the BIAcore assembly [27] yields a gel-phase volume of 0.11 nl in a 60-nl microchannel. On the grounds that ϕ is thus in the vicinity of 0.002, an estimate of 0.8 μM for $\left[\bar{\bar{X}}\right]$ [27] translates into a gel-phase concentration of 0.4 mM in the BIAcore system. Even greater disparities between $\left[\bar{\bar{X}}\right]$ and $[\bar{X}]_s$ occur in the IAsys system because of the much greater volume of liquid phase (typically 100 μl) in contact with the 2 nl gel phase (20 $mm^2 \times 100$ nm). Combination of the consequent estimate of $2 \cdot 10^{-5}$ for ϕ with a reported value of 3.2 nM for $\left[\bar{\bar{X}}\right]$ [22] signifies a $[\bar{X}]_s$ of 0.16 mM for the concentration of immobilized antibody sites within the gel phase of the IAsys cuvette. Thus, even though affinity site concentrations determined in the BIAcore instrument are typically 1000-fold higher than those obtained from IAsys studies, the actual concentrations within the gel phase are of the same order of magnitude.

In view of the uncertainty that surrounds the values of both ϕ and σ_A in biosensor studies, there seems to be little benefit gained from abandonment of the single-phase analysis in favour of that based on a biphasic system. Furthermore, retention of K_{AX} as the product of σ_A and $(K_{AX})_s$ as the characteristic binding constant has advantage that it is also the parameter evaluated from the ratio of rate constants determined by kinetic analysis of biosensor traces. Because the kinetic treatment is based on the same model of the ligate–matrix interaction as that used for the single-phase thermodynamic analysis ($A + X \rightleftarrows AX$), the parameters thus determined are necessarily the rate constants pertaining to the thermodynamic description of the reaction as a one-phase system. That explanation accounts for the essentially identical results reported for ligate interactions with affinity sites attached to a surface rather than a carboxymethyldextran gel [28]. Whereas those results [28] were taken to repudiate the existence of ligate at a lower concentration within the gel phase [11], they merely signify that the same means of expressing ligate concentration (that of the liquid phase) was used to interpret the results of experiments with and without gel phase.

4. Discussion

This development of a biphasic thermodynamic theory of quantitative affinity chromatography has served several purposes. (i) It has provided a more realistic thermodynamic model for analysis of results obtained by conventional partition equilibrium and frontal affinity chromatographic studies. (ii) It has established that the earlier binding parameter, K_{AX}, is the product of the ligate partition coefficient (σ_A) and the binding constant $(K_{AX})_s$ defined in terms of concentrations prevailing in the stationary phase. (iii) Similarly, the parameter formerly designated as $\left[\bar{\bar{X}}\right]$ in the single-phase theory is the product of the fractional stationary-phase volume (ϕ) and the total affinity site concentration within the stationary phase volume, $[\bar{X}]_s$. (iv) Although the biphasic theory is shown to be applicable also to results obtained by biosensor technology, its use with such data seems premature without (a) elucidation of the thickness of the sensor gel strip in order to allow evaluation of ϕ, and (b) an experimental method for measuring σ_A. In the interim, retention of the single-phase analysis is the preferred option because K_{AX}, the parameter thus determined, is also the quantity determined from the ratio of rate constants determined by current kinetic analyses.

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