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Review

Potential of biosensor technology for the characterization of interactions by quantitative affinity chromatography

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Abstract

This review places the characterization of interactions by biosensor technology in the broader context of their study by quantitative affinity chromatography. The general reluctance to consider biosensor-based characterization as a form of quantitative affinity chromatography on the grounds of a difference in aims of the two techniques reflects a mistaken belief that BIAcore and IAsys studies characterize the kinetics of the chemical reaction responsible for biospecific adsorption of a soluble reactant to an immobilized form of its affinity partner. It now transpires that the association and dissociation rate constants thereby determined refer to thermodynamic characterization of biospecific adsorption in terms of a single-phase model in which affinity sites are distributed uniformly throughout the liquid-phase volume accessible to the partitioning reactant—the model used for characterization of biospecific adsorption by quantitative affinity chromatography. In that light the most important attribute of biosensor technology is its potential for thermodynamic characterization of biospecific adsorption by virtue of its ability to monitor complex formation directly; and hence its potential for the characterization of interactions with affinities that are too strong for study by forms of quantitative affinity chromatography that monitor complex formation on the basis of reactant depletion from the liquid phase. Kinetic as well as thermodynamic analyses of biosensor data are described for attainment of that potential.

Keywords: Reviews; Biosensor technology; Protein–ligand interactions; Binding constants

Contents

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1. Introduction

Quantitative affinity chromatography [1–4] is a term used to describe techniques whereby equilibrium constants are determined from the concentration dependence of the extent of adsorption of a soluble solute (ligate) to an insoluble matrix as the result of biospecific interaction with affinity sites located on (within) the solid phase. As the sequel to successful deployment of affinity chromatography for solute purification [5], quantitative affinity chromatography was envisaged initially [1,6,7] as a column procedure for characterization of the equilibrium interactions responsible for the purification of a solute by preparative affinity chromatography – not only the interaction of ligate with matrix sites, but also the competitive ligand interaction responsible for its biospecific elution from the affinity matrix. However, the quantitative expressions were also modified to encompass direct determination of the partition equilibrium position by measuring the ligate concentration in the liquid phase of a slurry of affinity matrix containing a known total concentration of ligate [1,8–10].

Extension of the definition of quantitative affinity chromatography to include partition equilibrium studies enhanced greatly the versatility of the technique. Introduced initially as a means of characterizing interactions that were too weak for quantitative study by conventional means [1,8], the technique was then adapted to characterize interactions at the other end of the energy spectrum – those too strong for study by other procedures [11,12]. That endeavour was facilitated by the development of quantitative expressions in terms of the total concentration of competing ligand [13–15], their previous expression in terms of free ligand concentration being a restriction that necessitated establishment of its magnitude by equilibrium dialysis or the gel chromatographic counterpart thereof [11,12,16]. However, despite that advance, the available methods of quantitative affinity chromatography still relied upon ability to infer the concentration of complexed ligate from the difference between free and total ligate concentrations.

An alternative to deduction of the extent of complex formation from the concentration of ligate in the liquid phase is, of course, the direct measurement of complexed ligate – the principle involved in such techniques as filter-binding assays, solid-phase radioimmunoassays and enzyme-linked immunosorbent assays (ELISA). However, an unfortunate aspect of those methods is the need to remove the uncomplexed ligate by copious washing steps in order to assess the amount complexed with affinity sites on the solid phase. This disruption of the equilibrium state for measurement of an individual species concentration runs counter to a basic tenet of the law of mass action (Le Chatelier's principle), which signifies the necessity to make the concentration measurement in the unperturbed equilibrium mixture. Such neglect of this thermodynamic requirement, which is permissible in an operational sense, subject to demonstration that the rate of complex dissociation is sufficiently slow for negligible breakdown of complex to have occurred during the separation process [17], was dictated by an absence of methods for direct measurement of complexed ligate concentrations in equilibrium mixtures. The commercial development of suitable biosensor technology in the past decade [18,19] has provided that methodology – an advance that offers the prospect of extending the versatility of quantitative affinity chromatography to include the characterization of even stronger interactions.

Despite the fact that the expressions derived for

quantitative affinity chromatography also apply directly to results obtained by biosensor technology with either the BIAcore or IAsys instruments, the initial approach entailed the development of new theory for evaluation of the equilibrium constant as the ratio of association and dissociation rate constants inferred from the form of the progress curves for ligate adsorption to, and desorption from, affinity sites covalently attached to the sensor surface [20–22]. However, the kinetic analysis is proving to be more complicated than envisaged originally [23–27], whereupon the value of biosensor technology is the provision of results for the thermodynamic characterization of high-affinity interactions because of its ability to monitor the concentration of complexed rather than free ligate. In this review we first summarize the relevant aspects of quantitative affinity chromatography to set the scene for their extension to the characterization of interactions by biosensor technology – a term used rather than biospecific interaction analysis (BIA), which seems to have assumed purely kinetic connotations [28,29].

2. Quantitative affinity chromatography as a partition procedure

Although affinity chromatography is usually regarded as a column procedure, the parameter governing the retardation of solute (ligate, A) is the partition coefficient – the ratio of ligate concentrations in the stationary and liquid (mobile) phases. Evaluation of affinity constants by measuring the ligate distribution between the two phases thus provides a more direct form of quantitative affinity chromatography. The fact that partition equilibrium experiments may be considered in such terms is the avenue through which the study of interactions by biosensor technology becomes an integral part of quantitative affinity chromatography.

2.1. Simple partition equilibrium technique

In the simplest form of partition equilibrium experiment [1], a fixed amount of affinity matrix is placed in each of a series of tubes and equilibrated with a known volume of buffer, or of buffer containing a known concentration of a ligand (S) that competes with affinity matrix sites, X, for ligate (Fig. 1). Each slurry of affinity matrix is then supplemented with an aliquot of concentrated ligate solution (in the same buffer or ligand-supplemented buffer) to prepare a series of reaction mixtures with known total ligate concentrations, $[\overline{A}]$. After establishment of partition equilibrium, a sample of each liquid phase is obtained by centrifugation or filtration in order to determine $[\overline{A}]$, the concentration of ligate remaining in that phase. In competition experiments this liquid phase includes ligate–ligand complex(es) as well as free ligate: in the absence of competitor, $[\overline{A}]$ becomes synonymous with the free ligate concentration, [A]. This ability to define the concentration of ligate bound to affinity matrix sites, $([\overline{\overline{A}}] - [\overline{A}])$, as a function of its concentration in the liquid phase, $[\overline{A}]$, allows characterization of the interaction between ligate and matrix sites in terms of an effective equilibrium constant and a total concentration of matrix sites (see Section 3).

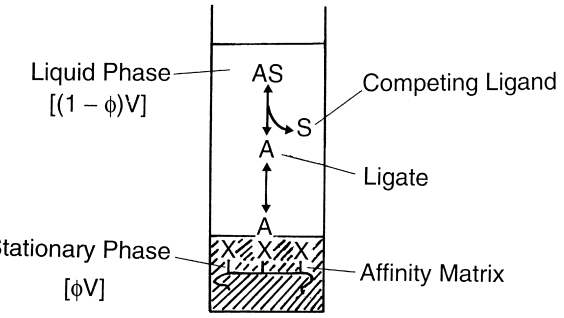

Fig. 1. Schematic representation of the simple partition variant of quantitative affinity chromatography in which stationary phase occupies a fraction ϕ of the total volume; and in which competing ligand S and immobilized affinity sites X compete for ligate A.

2.2. BIAcore-based measurement of partition equilibrium

In the BIAcore instrument a solution of ligate in buffer (or in buffer supplemented with competing ligand) flows through a microchannel, the base of which comprises a layer of affinity matrix attached to the sensor surface (Fig. 2a). Whereas the measurement of ligate distribution between phases by simple partition measurements entailed delineation of the matrix-bound ligate concentration as the difference between $[\overline{\overline{A}}]$ and $[\overline{A}]$, the biosensor response monitors the difference, $([\overline{\overline{A}}] - [[\overline{A}])$, directly on the basis of the increased refractive index of the matrix

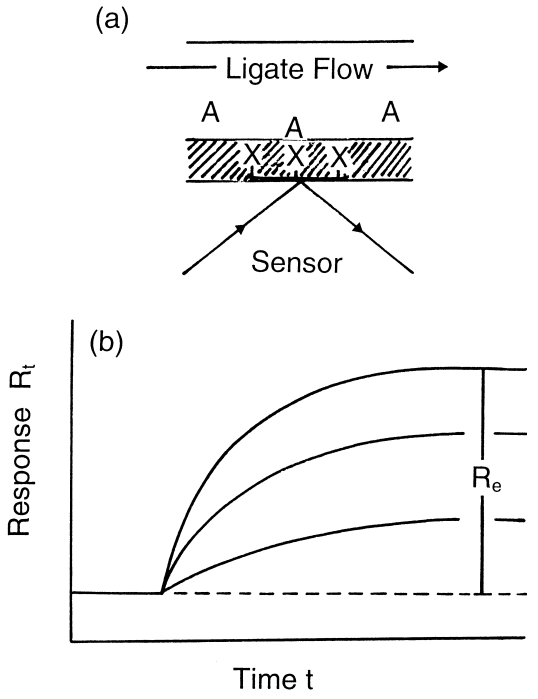

Fig. 2. Characterization of the interaction between a ligate and immobilized affinity sites by biosensor technology. (a) Schematic representation of the BIAcore microchannel sensor system (not to scale: the gel layer is miniscule in comparison with the liquid-phase height). (b) Form of the time dependence of biosensor response during the adsorption stage of the experiment.

layer due to ligate binding. Because the liquid phase is being replaced continually by fresh ligate solution, the composition of the liquid phase may be identified with that of the injected solution. A series of experiments with a range of injected ligate concentrations thus yields a series of sensorgrams (Fig. 2b) from which the dependence of the equilibrium response, R_e, as a function of $[\overline{A}]$ suffices for determination of the equilibrium constant and the maximum response, R_m ($\equiv[\overline{\overline{X}}]$, the total concentration of matrix sites).

2.3. Partition equilibrium as a stepwise titration procedure

The problem of ensuring the presence of identical amounts of affinity matrix in partition studies is overcome readily by switching to a recycling partition procedure (Fig. 3), in which the liquid phase

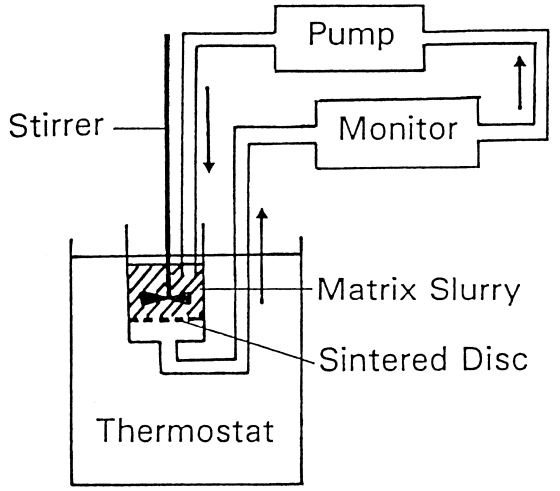

Fig. 3. The recycling partition equilibrium variant of quantitative affinity chromatography. Adapted from Ref. [13].

from a stirred slurry of affinity matrix passes through a ligate-monitoring device before being returned to the slurry [8,13]. After equilibration of the slurry with buffer (or buffer supplemented with competing ligand), the addition of an aliquot of ligate solution gives rise to a progressive change in the monitored ligate concentration in the liquid phase until a time-independent (equilibrium) value is attained. Having thus obtained one $\{[\overline{A}]-[\overline{A}]), [\overline{A}]\}$ combination, it is now possible to make further additions of concentrated ligate solution to generate the whole data set for characterizing the matrix–ligate interaction in the form of a stepwise titration.

Advantage may also be taken of an analogous stepwise titration format in biosensor studies with the IAsys instrument [30], the cuvette-based design of which is compatible with successive ligate additions. In this instrument the affinity matrix is attached to a sensor surface implanted in the base of a stirred cuvette [31]. Whereas recycling of the liquid phase was required in the above procedure to assess the attainment of partition equilibrium from time-independence of $[\overline{A}]$, the corresponding criterion of equilibrium attainment in the IAsys instrument is constancy of the refractometrically monitored concentration (R) of complexed ligate. Indeed, the form of the progress curve for complex formation is qualitatively similar to that shown in Fig. 2b; but in this instance the equilibrium response (R_e) refers to a

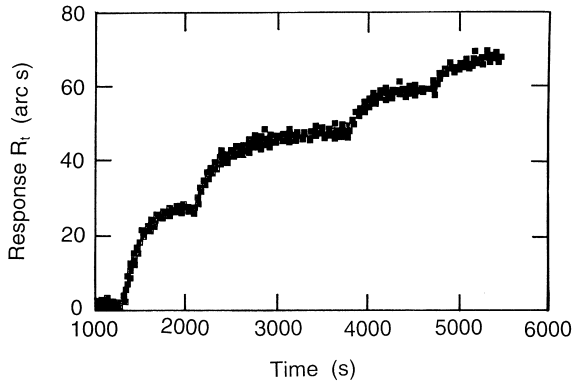

Fig. 4. Stepwise titration data obtained by adding successive aliquots of carboxypeptidase to an IAsys cuvette with elicited monoclonal antibody immobilized on the sensor surface. Adapted from Ref. [30].

liquid-phase ligate concentration, $[\overline{A}]$, that is smaller than its initial value ($[\overline{\overline{A}}]$) by an amount corresponding to R_e [30,32]. An example of the use of the IAsys assembly for the conduct of a stepwise titration is shown in Fig. 4, which refers to the interaction of carboxypeptidase A with an elicited monoclonal antibody immobilized on the sensor surface [30].

3. Basic quantitative expressions

Results obtained by quantitative affinity chromatography and its biosensor technology counterpart have usually been considered in terms of a model in which the ligate A is univalent in its interaction with matrix sites X (Eq. (1a)), 1:1 stoichiometry also being ascribed to the competing interaction with ligand S (Eq. (1b)).

$$A + X \overset{K_{AX}}{\rightleftarrows} AX \tag{1a}$$

$$A + S \overset{K_{AS}}{\rightleftarrows} AS \tag{1b}$$

K_{AX} and K_{AS} denote the association equilibrium constants for the respective interactions of ligate with affinity sites and competing ligand. Although affinity chromatography is a two-phase process, its description in terms of these two equations implies that both ligate and affinity sites are uniformly distributed throughout a single phase [1]. Albeit unrealistic in a physical sense, this model provides a valid thermodynamic description of the ligate distribution between the two phases.

3.1. Thermodynamic consideration as a single-phase system

Consider the situation in which a known amount of ligate (A) is introduced into a slurry of affinity matrix with accessible volume *V*. The total concentration of ligate, $[\overline{\overline{A}}]$, is obtained by dividing the amount of A present by *V*; and, although of unknown magnitude, there is also an effective total concentration of matrix affinity sites, $[\overline{\overline{X}}]$, a quantity to be deduced from the analysis. Measurement of the ligate concentration in the liquid phase, $[\overline{A}]$, thus allows determination of the concentration of complexed ligate as ($[\overline{\overline{A}}]-[\overline{A}]$) after establishment of partition equilibrium. A series of such experiments conducted with a range of total ligate concentrations, $[\overline{\overline{A}}]$, but the same total concentration of affinity sites ($[\overline{\overline{X}}]$ constant) yields a binding curve for the interaction of ligate with matrix sites. Nonlinear regression analysis of the $\{([\overline{\overline{A}}]-[\overline{A}]), [\overline{A}]\}$ data set in terms of the appropriate rectangular hyperbolic relationship, namely

$$([\overline{\overline{A}}] - [\overline{A}]) = K_{AX}[\overline{\overline{X}}][\overline{A}]/(1 + K_{AX}[\overline{A}]) \tag{2}$$

provides a means of evaluating K_{AX} and $[\overline{\overline{X}}]$. Alternatively, linear transforms of Eq. (2) such as the Scatchard equation [33]

$$([\overline{\overline{A}}] - [\overline{A}])/[\overline{A}] = K_{AX}[\overline{\overline{X}}] - K_{AX}([\overline{\overline{A}}] - [\overline{A}]) \tag{3}$$

or the double-reciprocal formulation,

$$1/([\overline{\overline{A}}] - [\overline{A}]) = 1/[\overline{\overline{X}}] + 1/(K_{AX}[\overline{\overline{X}}][\overline{A}]) \tag{4}$$

may be used for the same purpose. The dependence of $([\overline{\overline{A}}]-[\overline{A}])/[\overline{A}]$ upon $([\overline{\overline{A}}]-[\overline{A}])$ has a slope of $-K_{AX}$ and an abscissa intercept of $[\overline{\overline{X}}]$, whereas that of $1/([\overline{\overline{A}}]-[\overline{A}])$ upon $1/[\overline{A}]$ allows determination of the same parameters from the ordinate ($1/[\overline{\overline{X}}]$) and abscissa ($-K_{AX}$) intercepts. However, the use of such linear transforms is open to criticism because of its reliance upon transformed experimental parame-

ters and the consequent statistical distortion that results from such transformations [34–36].

In the presence of a competing ligand, S, the partition behaviour of ligate requires description in terms of both equilibria [Eq. (1a) and Eq. (1b)]. Nevertheless, the results are still amenable to interpretation as above [4], provided that the effective ligate–matrix binding constant evaluated by means of Eqs. (2)–(4) is regarded as a constitutive parameter, $\overline{K}_{AX}$, related to K_{AX} by the expression

$$Q = K_{AX}/\overline{K}_{AX} = 1 + K_{AS}[S] \quad (5)$$

where [S] denotes the free ligand concentration. The binding constant for the ligate interaction with competing ligand (K_{AS}) may thus be obtained from the slope of the linear dependence of the ratio of ligate matrix equilibrium constants upon the free ligand concentration in reaction mixtures to which each $\overline{K}_{AX}$ refers. In instances where the only quantity available is the total ligand concentration, $[\overline{S}]$, the free ligand concentration in Eq. (5) is eliminated by means of the relationship [13,15]

$$[S] = [\overline{S}] - (Q-1)[\overline{A}]/Q \quad (6)$$

The counterpart of Eq. (5) may thus be written in the form

$$Q = 1 + K_{AS}\{[\overline{S}] - (Q-1)[\overline{A}]/Q\} \quad (7)$$

whereupon the equilibrium constant for the competing interaction is obtained from the dependence of the ratio of ligate–matrix equilibrium constants (Q) upon $\{[\overline{S}]-(Q-1)[\overline{A}]/Q\}$.

3.2. An alternative two-phase thermodynamic analysis

Although the above description of the two-phase interaction in single-phase terms suffices for thermodynamic purposes, an alternative two-phase characterization has been developed [37] in response to the fact that users of biosensor technology are attempting to ascribe mechanistic significance to the parameters evaluated by such means. That development allows comparisons to be drawn between the magnitudes of parameters determined by thermodynamic analysis in terms of single-phase and two-phase models. To simplify notation for the comparison we restrict consideration to an experiment conducted in the absence of competing ligand so that the concentration of ligate in the liquid phase equates with its free concentration ($[\overline{A}]=[A]$).

Consider the situation (Fig. 1) in which the stationary (gel) phase comprises a fraction ϕ of the total volume V. Because the affinity sites are restricted to the stationary phase volume, ϕV, they are ascribed a total concentration $[\overline{X}]_s$, where the subscript has been added to denote the phase. On the other hand, the ligate is distributed throughout both phases, its concentration in the stationary phase, $[A]_s$, being related to its liquid phase concentration, [A], by

$$[A]_s = \sigma_A[A] \quad (8)$$

where σ_A is the partition coefficient for ligate. Interaction between ligate and affinity sites is necessarily confined to the stationary phase, and hence the concentration of bound ligate is expressed as

$$([\overline{A}]_s - [A]_s) = (K_{AX})_s[\overline{X}]_s[A]_s/\{1 + (K_{AX})_s[A]_s\} \quad (9)$$

where $[\overline{A}_s]$ denotes the total ligate concentration (A and AX) within the stationary phase, and $(K_{AX})_s$ is the binding constant when concentrations are expressed in terms of those in that phase.

On the basis of direct proportionality between biosensor response, R_e, and the concentration of complexed ligate, the counterpart of Eq. (9) for the BIAcore instrument may be written

$$R_e = R_m(K_{AX})_s\sigma_A[A]/\{1 + (K_{AX})_s\sigma_A[A]\} \quad (10)$$

where R_m is the total concentration of affinity sites expressed in terms of instrument response (equivalent to $[\overline{X}]_s$ in Eq. (9)); and where Eq. (8) has been used to express $[A]_s$ in terms of ligate concentration in the liquid phase – the experimentally available parameter. The parameters derived from the rectangular hyperbolic dependence of R_e upon [A] are thus the matrix capacity for ligate, R_m, and the $\sigma_A(K_{AX})_s$, whereas the corresponding analysis in terms of single-phase theory yields R_m and K_{AX}: the two equilibrium constants are thus inter-related by the partition coefficient.

Although the concentration of bound ligate is also monitored in the IAsys instrument, the experimental-

ly available ligate concentration is $[\overline{\overline{A}}]$; and hence a mass conservation argument needs to be used to deduce the magnitude of [A] from the initial amount of ligate introduced into the cuvette, $V[\overline{A}]$, and the amount of complexed ligate, the relevant expression being [37]

$$V[\overline{\overline{A}}] = \{(1-\phi)V\}[A] + (\phi V)\sigma_A[A] + (\phi V)(R_e/F) \quad (11)$$

where F is the proportionality constant between instrument response and bound ligate concentration. The concentration of ligate in the liquid phase may thus be determined as

$$[A] = \{[\overline{\overline{A}}] - \phi(R_e/F)\}/\{1-\phi(1-\sigma_A)\} \quad (12)$$

which takes rigorous account of the fact that the formation of complex between ligate and matrix affinity sites is at the expense of the ligate concentration in the liquid phase [30,32,37]. In as much as IAsys measurements are normally interpreted (see, e.g., Ref. [38]) in terms of Eq. (11) with $[\overline{\overline{A}}]$ substituted for [A], such characterization is seen to be based on the premise that that $\phi(R_e/F) \ll [\overline{\overline{A}}]$, the assumed value of unity for the denominator in Eq. (12) being justified by the small magnitude of ϕ (see below). However, as noted elsewhere [30,32,37], the validity of this approximation needs close examination; and resort made to the exact expression in instances where the approximation is demonstrably invalid.

Although Eq. (10) provides, in principle, a means of determining the operative binding constant under conditions pertinent to the gel phase, the evaluation of $(K_{AX})_s$ presupposes an ability to specify the magnitude of σ_A, the ligate partition coefficient. On the basis of a random-fibre model [39] of the carboxymethyldextran gel, Schuck [24] has derived the expression $\sigma_A = \exp(-0.000638M_A^{2/3})$ for the partition coefficient of a spherical protein ligate with molecular weight M_A. On that basis the partition coefficient of a protein ligate with a molecular weight of 50 000 would be in the vicinity of 0.4, which signifies the likelihood of a two- to three-fold disparity between K_{AX} and $(K_{AX})_s$.

In the absence of experimental methods for verifying the magnitude of σ_A, there seems to be little point in discarding the analysis in terms of single-phase theory. Indeed, retention of K_{AX}, the product of σ_A and $(K_{AX})_s$, as the characteristic of binding strength has the advantage that it is also the parameter that is evaluated from the ratio of rate constants determined by kinetic analysis [20–22]. Because that treatment is also based on Eq. (1a) as the model of the interaction between ligate and matrix sites, the parameters thereby determined are necessarily the rate constants pertaining to the thermodynamic description of the reaction as a single-phase system [37]. That realization also explains the essentially identical results reported for ligate interactions with affinity sites attached to a surface rather than a carboxymethyldextran gel [31,40]. Whereas the latter results [40] were taken to repudiate the Schuck assertion [24] that σ_A should be significantly smaller than unity, they do not comment on that proposition. Instead, they merely signify identical means of expressing ligate concentration (that of the aqueous phase) in the series of experiments with and without gel phase (see Section 3.4). Furthermore, although the use of a relatively small ligate and low concentrations of immobilized ligand precludes any test of the additional prediction [24] of potential mass transport restrictions within the gel phase, it also illustrates a means of avoiding any such problems.

3.3. Allowance for multivalence of the partitioning ligate

In the characterization of biospecific interactions by biosensor technology the selection of the larger reactant as ligate is advantageous from the viewpoint of greater sensitivity of complex detection in molar terms. For this reason, many studies of immunological reactions have entailed the interaction of monoclonal antibody with immobilized antigen [20,21,41–43]. Such studies now tend to be conducted with the Fab fragment of the antibody [25,44–46] to comply with the requirement for univalence of ligate that is inherent in the kinetic analysis. On the other hand, the use of multivalent ligates is widespread in research areas such as the study of protein–carbohydrate interactions by biosensor technology [47–51], and the study of lipid–protein interactions with phospholipid micelles as ligate [52]. Steps are under

way to incorporate correct allowance for ligate bivalence in the kinetic analysis.

Multivalence of the ligate is readily incorporated into the thermodynamic analysis of biosensor data [53], the counterpart of Eq. (3) for an f-valent ligate being

$$([\overline{\overline{A}}]^{1/f} - [\overline{A}]^{1/f})/[\overline{A}]^{1/f}) = K_{AX}[\overline{\overline{X}}] - fK_{AX}[\overline{\overline{A}}]^{(f-1)/f}([\overline{\overline{A}}]^{1/f} - [\overline{A}]^{1/f}) \quad (13a)$$

where K_{AX} denotes the intrinsic binding constant [54] for equivalent and independent interactions between ligate and affinity sites. Evaluation of the binding constant by means of this multivalent counterpart of the Scatchard analysis [55,56] thus requires determination of the slope $(-fK_{AX})$ of the linear dependence of $([\overline{\overline{A}}]^{1/f} - [\overline{A}]^{1/f})/[\overline{A}]^{1/f})$ upon $[\overline{\overline{A}}]^{(f-1)/f}([\overline{\overline{A}}]^{1/f} - [\overline{A}]^{1/f})$. Alternatively, any bias of experimental uncertainty introduced by use of a linear transform is eliminated by writing Eq. (13a) as

$$f([\overline{\overline{A}}]^{1/f} - [\overline{A}]^{1/f})[\overline{\overline{A}}]^{(f-1/f)} = K_{AX}[\overline{\overline{X}}] \times [\overline{\overline{A}}]^{(f-1)/f}[\overline{A}]^{1/f}/\{1 + fK_{AX}[\overline{\overline{A}}]^{(f-1)/f}[\overline{A}]^{1/f}\} \quad (13b)$$

whereupon $f([\overline{\overline{A}}]^{1/f} - [\overline{A}]^{1/f})[\overline{\overline{A}}]^{(f-1)/f}$ exhibits a rectangular hyperbolic dependence upon $[\overline{A}]^{(f-1)/f}[\overline{A}]^{1/f}$ [56].

Because the total and liquid-phase concentrations of ligate need to be raised to a power other than unity, the substitution of instrumental response R_e for the bound ligate parameter is no longer tenable. Instead, advantage needs to be taken of the expression

$$[\overline{\overline{A}}] = [\overline{A}] + R_e/(FM_A) \quad (14)$$

where $F = 60\,000$ units l g^{-1} for proteins [53] in the BIAcore instrument: the corresponding value for the IAsys biosensor is 815 000 arc s l g^{-1} for a cuvette containing 100 μl solution, but requires division by the ratio $(100/V)$ to accommodate a different volume V (μl) of liquid phase [30]. Because of the different specific refractive increment for nucleic acids, the values of F are four-fifths of those stated above in studies with an oligonucleotide as ligate [57]. In BIAcore studies Eq. (14) is used to calculate $[\overline{A}]$ from the injected ligate concentration $[\overline{\overline{A}}]$ and R_e, whereas it provides the means of obtaining $[\overline{\overline{A}}]$ from $[\overline{A}]$ and R_e in IAsys studies.

In principle, the appropriate value of ligate valence (f) is required to obtain a linear multivalent Scatchard plot. However, there are interactions for which the plot of results according to the conventional Scatchard formulation (Eq. (3); i.e., Eqs. (13a) and (13b) with $f = 1$] exhibits linearity despite multivalence of the ligate [53]. This situation arises when $([\overline{\overline{A}}] - [\overline{A}])/[\overline{A}] \ll 1$, an approximation that allows advantage to be taken of the binomial theorem to simplify Eq. (13a) to the form [53]

$$([\overline{\overline{A}}] - [\overline{A}])/[\overline{A}] = fK_{AX}[\overline{\overline{X}}] - fK_{AX}([\overline{\overline{A}}] - [\overline{A}]) \quad (15)$$

Thus, provided that the concentration of ligate in the liquid phase greatly exceeds its concentration in the form of ligate–matrix complex, the equilibrium responses are amenable to standard Scatchard analysis, except that the linear dependence of $([\overline{\overline{A}}] - [\overline{A}])/[\overline{A}]$ upon $([\overline{\overline{A}}] - [\overline{A}])$ has a slope of $-fK_{AX}$. Alternatively, analysis in terms of a rectangular hyperbolic dependence of $([\overline{\overline{A}}] - [\overline{A}])$ upon $[\overline{A}]$ yields fK_{AX} and $[\overline{\overline{X}}]$ as the two curve-fitting parameters.

In the sense that the approximation entailed in the derivation of Eq. (15) from Eqs. (13a) and (13b) is likely to be valid for a great many systems studied by biosensor technology, Eq. (15) undoubtedly provides a logical basis for observations of seeming compliance with 1:1 stoichiometry in biosensor studies of interactions for which an assumed valence of unity for ligate is highly suspect. Although developed specifically from considerations of thermodynamic studies, the same conclusion applies also to kinetic analysis of biosensor traces conforming with the pseudo-first-order behaviour symptomatic of 1:1 stoichiometry (see below). The equilibrium constants reported for the interactions of lectins with immobilized carbohydrate residues [47–52] are therefore likely to be the product fK_{AX}, reflecting the combination of an evaluated dissociation rate constant with a parameter that was actually the product of the lectin valence and the association rate constant for the interaction between lectin and immobilized affinity sites. However, difficulties arise in such studies when the concentration of immobilized sites becomes sufficiently high to invalidate the approximation that $([\overline{\overline{A}}] - [\overline{A}])/[\overline{A}]$ is small [43,53].

3.4. Kinetic approach to the evaluation of binding constants

As noted above, the kinetic approach [20–22] to characterizing the interaction of ligate with immobilized affinity sites is also based on 1:1 interaction (Eq. (1a)), the equilibrium constant for which (K_{AX}) is expressed as the ratio of association (k_a) and dissociation (k_d) rate constants. That this approach is also based on single-phase thermodynamic description of the interaction becomes evident from the differential equation used to express the rate of complex formation, namely,

$$d[AX]/dt = k_a[\overline{A}][X] - k_d[AX] \quad (16a)$$

where $[\overline{A}]$ is the concentration of ligate in the aqueous phase ($[\overline{A}]=[A]$ in an experiment with no competing ligand). The free concentration of affinity sites is eliminated by its expression as the difference between [AX] and $[\overline{X}]$, the effective total concentration of matrix sites; but the latter is merely a curve-fitting parameter to be determined from kinetic analysis of the time-dependence of AX formation. Upon substituting instrumental responses for concentrations of complexes, Eq. (16a) becomes

$$dR/dt = k_a R_m[\overline{A}] - (k_a[\overline{A}] + k_d)R \quad (16b)$$

whereupon the concentration dimension of the association rate constant (M^{-1} s^{-1}) depends solely upon the scale in which the free ligand concentration is described. In other words, the concentration units of $[\overline{X}]$ and [AX] (whether expressed as such or as respective instrumental responses R_m and R) are irrelevant in the sense that they self-cancel. Consequently, the value of R_m (or $[\overline{X}]$) derived from the analysis is based on the concentration scale used to define k_a. On the grounds that the affinity sites have therefore, by default, been considered to be distributed in the same volume as ligate, the kinetic approach is also defining the single-phase thermodynamic model.

In the BIAcore instrument the continual flow of ligate solution across the affinity matrix is taken to ensure constancy of the liquid-phase ligate concentration, $[\overline{A}]$, whereupon Eqs. (16a) and (16b) become a pseudo-first-order kinetic expressions with solution [22]

$$R_t = R_m k_a[\overline{A}]\{1 - \exp(-k_{obs}t)\}/k_{obs} \quad (17a)$$

$$k_{obs} = (k_a[\overline{A}] + k_d) \quad (17b)$$

An alternative relationship, derived [53,58] on the basis of the same model and approximation, expresses the time dependence of instrumental response (R_t) in terms of the equilibrium (R_e) rather than maximal (R_m) response (Eq. (18)).

$$(R_e - R_t) = R_e \exp(-k_d t) \quad (18)$$

Either kinetic analysis of the adsorption stage of an experiment yields a pseudo-first-order rate constant, k_{obs}, that is related to the injected free ligate concentration $[\overline{A}]$ by Eq. (17b). In principle, the linear dependence of k_{obs} upon $[\overline{A}]$ therefore provides values of k_a and k_d from the slope and ordinate intercept, respectively. Furthermore, subject to the validity of the approximation that the flow of buffer across the affinity matrix renders $[\overline{A}]$ zero during ligate desorption, the corresponding analysis of that stage of an experiment in terms of the expression

$$R_t/R_o = \exp(-k_{obs}t) \quad (19)$$

provides an independent estimate of the dissociation rate constant from the exponential decay of biosensor response expressed relative to its value (R_o) at the commencement of buffer flow across the sensor surface.

Although the same pseudo-first-order kinetic procedure has been recommended for the analysis of IAsys data with total ligate concentration substituted for its free conterpart in Eqs. (17a) and (17b) [31,38], such action is clearly predicated upon negligibility of the contribution of the $R/(FM_A)$ term to the right-hand side of Eq. (14). In the event that this identification of $[\overline{A}]$ with [A] in an experiment with no competing ligand ($[\overline{A}]=[A]$) ceases to be a valid approximation, the progress curve needs to be analyzed in terms of second-order kinetics to accommodate the progressive decrease in $[\overline{A}]$: a procedure based on numerical integration of Eq. (16b) has been developed for that purpose [30,32].

4. Illustrative thermodynamic analyses of biosensor data

Having summarized the theory of quantitative affinity chromatography in the context of biosensor

technology, we begin illustrating features of the various analyses by considering the first major BIAcore investigation to deviate from the kinetic approach.

4.1. BIAcore results for a univalent ligate

In an investigation of the interaction between immobilized interleukin-6 and a soluble form of its biospecific receptor [59], the decision to immobilize the cytokine reflected its dimeric state, and hence preclusion from consideration as a univalent ligate for purposes of conventional kinetic analysis. Thus, even though biological considerations suggest a reversal of roles, the monomeric and hence univalent receptor is the ligate for interaction with an affinity matrix comprising interleukin-6 covalently linked to the carboxymethyldextran layer on a biosensor chip. The results of flowing a range of concentrations of receptor across the cytokine-modified biosensor chip are presented as a Scatchard plot in Fig. 5a, where the line is the best-fit description obtained by non-linear regression analysis of the untransformed (R_e, $[\bar{A}]$) data in accordance with Eq. (2). This best-fit description signifies values of $2.4(\pm 0.2)\times 10^7\ M^{-1}$ and 2200($\pm$100) response units for the ligate–matrix binding constant (K_{AX}) and maximal matrix capacity (R_m), respectively. In keeping with the above demonstration that the conventional kinetic analysis [20–22] also refers to the single-phase model used for thermodynamic characterization, a comparable value of $5.6(\pm 1.2)\times 10^7\ M^{-1}$ emanates from the ratio of k_a ($4.5(\pm 0.5)\times 10^5\ M^{-1}\ s^{-1}$) and k_d ($8.0(\pm 0.8)\times 10^{-3}\ s^{-1}$) obtained by analyzing the progress curves in terms of Eqs. (17a) and (17b).

In order to obtain an equilibrium constant for the cytokine–receptor interaction in solution, receptor solutions supplemented with interleukin-6 were flowed across the cytokine-modified biosensor chip to obtain the constitutive binding constant $\bar{K}_{AX}$ for interpretation in terms of Eq. (7). However, the bivalence of the interleukin-6 dictated the modification of Eq. (7) to accommodate the fact that the concentration of competing ligand sites was twice the molar concentration of soluble cytokine. The consequent evaluation of K_{AS} is shown in Fig. 5b,

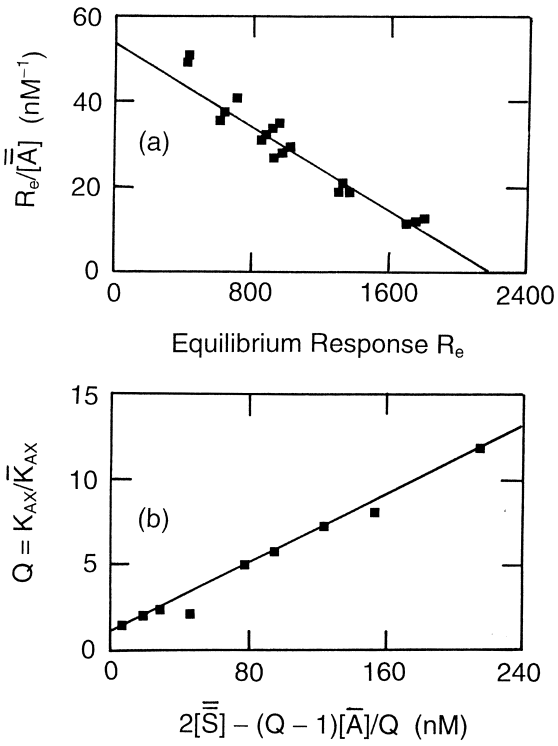

Fig. 5. Thermodynamic characterization of the interaction between interleukin-6 and the soluble form of its biospecific receptor by BIAcore technology. (a) Scatchard plot of data for the interaction of receptor with immobilized interleukin-6 sites on the biosensor chip. (b) Evaluation of the binding constant for the competitive interaction between receptor and interleukin-6 in solution. Adapted from Ref. [59].

from which the slope yields a binding constant of $4.8(\pm 0.3)\times 10^7\ M^{-1}$ [59]. In that regard it is possibly relevant that the two-fold difference between K_{AS} and K_{AX} would correlate reasonably well with the identification of K_{AX} as $\sigma_A(K_{AX})_s$ for a ligate of this size (see Section 3.2).

4.2. BIAcore results for a bivalent ligate

A second thermodynamic analysis of results obtained with the BIAcore instrument is included to illustrate various aspects of the theory relating to ligate multivalence, a study of the interaction be-

tween concanavalin A and the carboxymethyldextran layer on an unmodified biosensor chip [53] being used for this purpose. The application of Eq. (14) to obtain $[\bar{\bar{A}}]$ from $[\bar{A}]$ (=[A]) and the equilibrium response is summarized in Table 1; and analysis of the consequent ($[\bar{\bar{A}}]$, $[\bar{A}]$) data set according to Eq. (13b) on the basis of lectin bivalence ($f=2$) presented in Fig. 6a. Values of $2.52(\pm0.06)\times10^5\ M^{-1}$ and $0.86(\pm0.05)\ \mu M$ are obtained for the intrinsic binding constant (K_{AX}) and effective total concentration of matrix sites ($[\bar{\bar{X}}]$), respectively.

Inspection of the final column of Table 1 reveals that the magnitude of $([\bar{\bar{A}}]-[\bar{A}])/[\bar{A}]$ is sufficiently small to encourage the application of Eq. (15) to obtain the product $2K_{AX}$ from traditional analysis of the rectangular hyperbolic dependence of bound ligate upon the concentration of ligate in the liquid phase. Such treatment (Fig. 6b) yields magnitudes of 2610(±290) response units for R_m ($[\bar{\bar{X}}]=0.82\ \mu M$) and $3.4(\pm1.0)\times10^5\ M^{-1}$ for K_{AX}. However, the larger uncertainty in this estimate of K_{AX} reflects systematic departure of experimental points from the best-fit description due to progressively poorer conformity with the inherent approximation that $([\bar{\bar{A}}]-[\bar{A}])/[A]\ll1$ as $[\bar{A}]$ decreases (final column of Table 1). This demonstration of seemingly reasonable compliance with behaviour symptomatic of 1:1 interaction reinforces the point made earlier (Section 3.3) that the conformity with pseudo-first-order kinetic behaviour inferred from BIAcore studies of interactions between saccharidic affinity matrices and other lectins [47–49] does not necessarily justify their analysis in terms of 1:1 stoichiometry: the

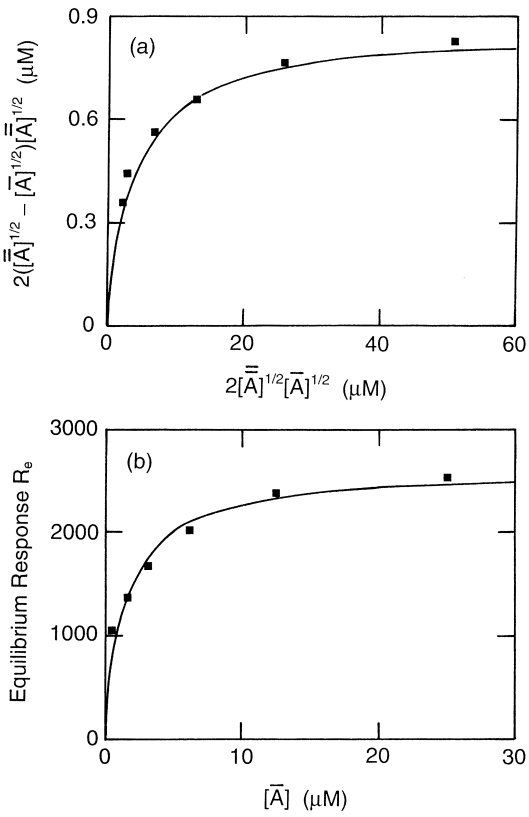

Fig. 6. Thermodynamic characterization of the interaction between concanavalin A (a bivalent ligate) and immobilized carboxymethyldextran by BIAcore technology. (a) Multivalent analysis (Eq. (13b)) of results for the dependence of equilibrium response upon injected lectin concentration (Table 1). (b) Corresponding analysis according to Eq. (15), which is subject to the assumption that $([\bar{\bar{A}}]-[\bar{A}])/[\bar{A}]\ll1$. Adapted from Ref. [53].

Table 1
Evaluation of the effective total concentration of ligate $[\bar{\bar{A}}]$ from the liquid-phase concentration ($[\bar{A}]$) and equilibrium BIAcore response (R_e) in studies of the interaction between concanavalin A and immobilized carboxymethyldextran[a]

$[\bar{A}]$ (μM)	R_e (units)	$([\bar{\bar{A}}]-[\bar{A}])$ (μM)[b]	$[\bar{\bar{A}}]$ (μM)	$([\bar{\bar{A}}]-[\bar{A}])/[\bar{A}]$
25.0	2544	0.82	25.82	0.03
12.5	2387	0.76	13.26	0.06
6.25	2010	0.64	6.89	0.09
3.13	1672	0.54	3.67	0.15
1.56	1370	0.44	2.00	0.22
0.78	1030	0.33	1.11	0.30

[a]Data taken from Ref. [53].
[b]Calculated as $R_e/(FM_A)$ with $F=60\,000$ and $M_A=52\,000$ [53].

reported equilibrium constants may well represent the product fK_{AX}.

4.3. Analysis of results obtained with the IAsys instrument

A distinctive feature of the thermodynamic analysis of results obtained by IAsys biotechnology is the need to take into account the fact that complex formation on the biosensor surface is at the expense of ligate concentration in the liquid phase [30]. To illustrate the importance of this consideration, we reinterpret results from an IAsys study [38] in which the abscissa of the rectangular hyperbolic dependence of binding response upon ligate concentration (Eq. (2)) was expressed in terms of $[\underline{\underline{A}}]$ instead of $[\underline{A}]$, the depleted concentration. The data reported in Fig. 8 of Ref. [38] for the interaction of recombinant chymotrypsin inhibitor 2 ($M_A = 9200$) with immobilized α-chymotrypsin are presented (○) in Fig. 7, where the retention of bound ligate concentration in terms of instrument response reflects univalence of the chymotrypsin inhibitor. Also shown (●) are the corresponding results after calculation of the ligate concentration in the liquid phase ($[\underline{A}]$) from Eq. (14) with $F = 1\,630\,000$ arc s l g^{-1} for this experiment with 200-μl samples of ligate solution. Nonlinear regression analysis of the revised data in terms of Eq. (2) increases the evaluated binding constant (K_{AX}) from 5.1×10^7 to 8.6×10^7 M^{-1}. An important aspect of this reassessment is the fact that the smallest response (7 arc s) reflects removal of only 0.47 n*M* ligate from the liquid phase, but an extent of depletion accounting for half of the ligate in the mixture under examination ($[\underline{\underline{A}}] = 0.97$ n*M*). Inasmuch as the range of ligate concentration examined is governed by the magnitude of K_{AX}, the allowance for ligate depletion becomes extremely important in the characterization of high-affinity interactions.

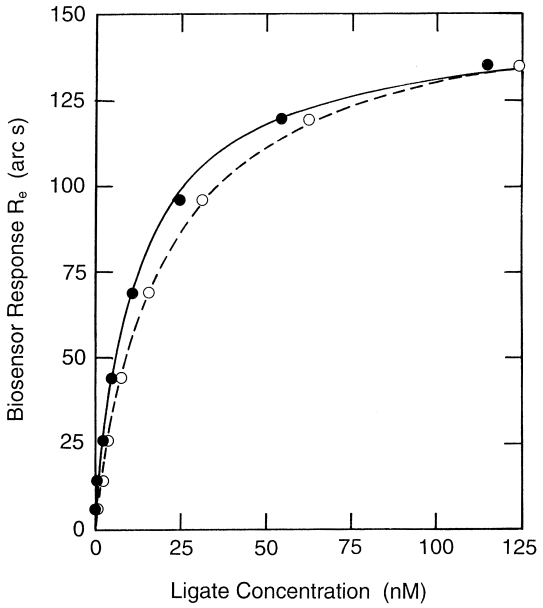

Fig. 7. Effect of ligate depletion of the liquid phase on the thermodynamic analysis of IAsys data for the interaction of recombinant inhibitor 2 with immobilized α-chymotrypsin: (○) data taken from Fig. 8 of Ref. [38]; (●) corresponding data after allowance for ligate removal from the liquid phase as the result of complex formation. Broken and solid lines denote the respective rectangular hyperbolic dependences with $[\underline{\underline{A}}]$ and $[\underline{A}]$ as the abscissa parameter.

5. Illustrative kinetic evaluation of biosensor data

The fact that the characterization of interactions by biosensor technology was introduced as a kinetic approach [20–22] has led to a situation wherein kinetic analyses predominate over their thermodynamic counterparts. From a mechanistic viewpoint, any description of the time-dependence of biosensor response must clearly include allowance for additional factors, such as the kinetics and thermodynamics of partition into the affinity matrix [24,60], the constancy or otherwise of ligate concentration throughout the bulk-liquid phase [23,61,62], and of ligate across the stagnant layer [23,61], before meaningful rate constants for the chemical kinetics of the interaction between ligate and immobilized affinity sites can be obtained. However, in view of the realization that the kinetic characterization procedures adopted merely provide phenomenological rate constants for the single-phase thermodynamic description of the interaction (Section 3.2), the most important factor to consider is the possibility that the

time dependence of response is being dominated by mass-transport limitations in the liquid phase [23,61–63]. Independence of the time-course of binding response upon the flow-rate used for its generation is the recommended procedure for justifying the neglect of mass-transport effects in the kinetic analysis [23,24,28,61].

5.1. Simple pseudo-first-order kinetic analysis

Kinetic characterization of interactions by BIAcore technology is generally based on the premise that the concentration of free ligate may be equated with that of the injected solution, thereby rendering the characterization of a 1:1 interaction a simple pseudo-first-order kinetic analysis. Initial applications of this procedure [20,21] relied upon interpretation of the time-courses of biosensor response in terms of the differential rate expression (Eq. (16b)), the magnitude of k_{obs} being obtained from the slope of the linear dependence of dR/dt upon R – a procedure depicted schematically in Fig. 8. However, it was soon realized that analysis of the data in terms of an integrated form of the rate expression (Eqs. (17a) and (17b) or Eq. (18)) afforded a more critical appraisal of their conformity with the pseudo-first-order behaviour commensurate with 1:1 interaction [22,23].

A BIAcore study of the interaction between the Fab fragment of a monoclonal anti-paraquat antibody and an immobilized derivative of its eliciting antigen [25] is presented in Fig. 9 to illustrate the simple pseudo-first-order kinetic analysis currently adopted. Experimental data from the adsorption stage of the biosensor trace generated by the passage of 4 μM Fab across the antigen-modified biosensor chip are shown (●) in Fig. 9a, together with their best-fit description in terms of Eq. (18) with R_e assigned the magnitude of the plateau (equilibrium) response. Values of the consequent pseudo-first-order rate constant (k_{obs}) and those from a series of such experiments with a range of injected ligate concentrations ([$\underline{A}$]) are plotted according to Eq. (17b) in Fig. 9b, the slope and ordinate of which yield estimates of $9.7(\pm 0.6)\times 10^4$ M^{-1} s^{-1} and $0.032(\pm 0.017)$ s^{-1} for k_a and k_d, respectively: a binding constant (K_{AX}) of $3.0(\pm 1.8)\times 10^6$ M^{-1} is then obtained from their ratio.

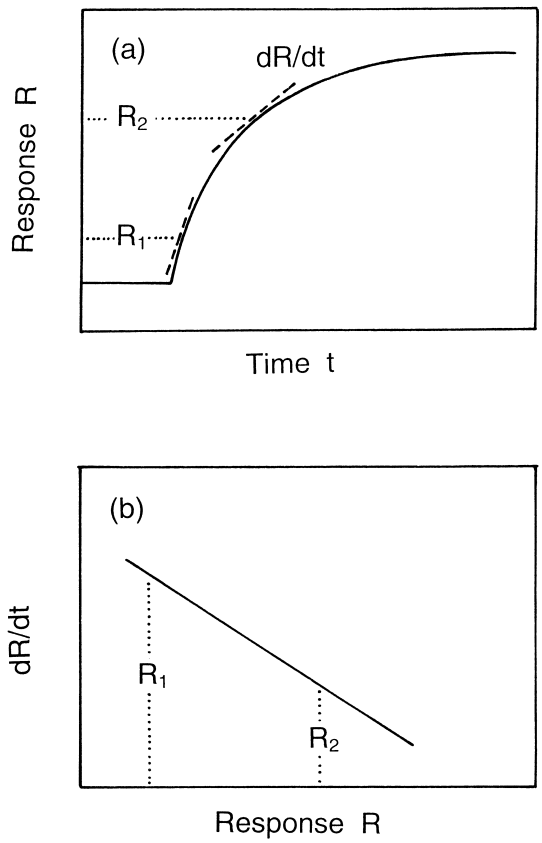

Fig. 8. Illustration of the initial procedure devised for kinetic characterization of interactions by BIAcore technology. (a) Estimation of tangents to the progress curve at a series of biosensor responses. (b) Plot of the dependence of those values of dR/dt upon R for the evaluation of k_{obs} ($=k_a[\underline{A}]+k_d$) from the slope.

5.2. Deviation from predicted behaviour for 1:1 interactions

The switch to analysis in terms of the integrated rate equations quickly led to the realization that results at variance with pseudo-first-order kinetic behaviour were being obtained in many studies of interactions for which an assumed 1:1 stoichiometry was entirely reasonable [22,25,30,31,38,64–69]. Vari-

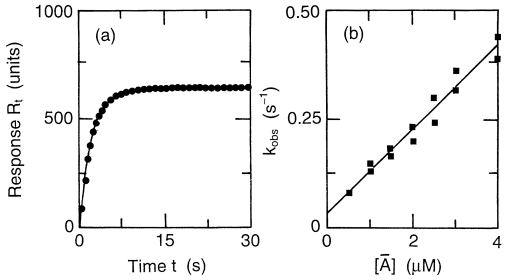

Fig. 9. Kinetic analysis of data reflecting passage of the Fab fragment of an anti-paraquat monoclonal antibody through the microchannel of a BIAcore assembly with paraquat immobilized on the sensor chip. (a) Adsorption stage of the progress curve for 4 μ*M* Fab, together with the best-fit description in terms of Eq. (18). (b) Use of the dependence of the pseudo-first-order rate constant (k_{obs}) upon injected ligate concentration to evaluate the association and dissociation rate constants via Eq. (17b). Adapted from Ref. [25].

ous explanations have been put forward to account for the seemingly anomalous kinetic behaviour.

(i) One possible explanation is heterogeneity of sites within the affinity matrix [64,65,69], whereupon the binding response requires description in terms of the relationships

$$R_t = \sum_{i=1}^{n} (R_m)_i (k_a)_i [\bar{A}]_i \{1 - \exp[-(k_{obs})_i t]\}/(k_{obs})_i \quad (20)$$

$$R_t = \sum_{i=1}^{n} (R_e)_i \{1 - \exp[-(k_{obs})_i t]\} \quad (21)$$

instead of Eq. (17a) and Eq. (18), respectively. Although the deviation from pseudo-first-order kinetic behaviour can usually be described as the sum of two exponentials ($n=2$ in Eq. (20) and Eq. (21)), there is no reason, a priori, for restricting the summations in these expressions to two classes of affinity site [67,68].

(ii) An alternative chemical explanation of such behaviour entails modification of the mechanism to include additional reactions such as isomerization of the AX complex to a state AX* [57,66,67], whereupon there is further complex formation to restore equilibrium between A, X and AX. Thus the reaction scheme (Eq. (1a)) is extended to become

$$A + X \underset{k_d}{\overset{k_a}{\rightleftarrows}} AX \underset{k_r}{\overset{k_f}{\rightleftarrows}} AX^* \quad (22)$$

in which the isomerization step is governed by equilibrium constant $Y = k_f/k_r$. Taken in conjunction with the expression for conservation of affinity sites, $[\bar{X}] = [X] + [AX] + [AX^*]$, and the corresponding expression for conservation of ligate if the IAsys instrument is being used, the differential equations for this reaction scheme, namely,

$$d[AX]/dt = k_a[\bar{A}][X] - \{k_d + k_f\}[AX] + k_r[AX^*] \quad (23a)$$

$$d[AX^*]/dt = k_f[AX] - k_r[AX^*] \quad (23b)$$

are readily solved by numerical integration procedures to obtain the best-fit description of a given time-course of biosensor response [25,67]. That this model can be extended to include a series of post-binding events is evident from an experimental study of the interaction between ETS1 recombinant oncogene proteins and purine-rich deoxyoligonucleotides attached to the biosensor surface [57]. A model incorporating several polymerization as well as isomerization steps was invoked to account for the forms of the time courses of biosensor response in the adsorption and desorption stages of each experiment.

(iii) A third type of explanation involves the concept of restricted ligate access to affinity sites, either as the result of mass-transport limitations [23,24,60–63], or as the result of temporary unavailability of sites – the so-called parking problem [68,70].

(iv) In the IAsys instrument there is also the possibility that the deviation from pseudo-first-order kinetic behaviour may merely reflect the systematic depletion of free ligate concentration as the result of complex formation [30,32]. That situation, which signifies invalidity of the pseudo-first-order kinetic assumption rather than nonconformity with the behaviour of a 1:1 interaction, necessitates the introduction of a second-order kinetic analysis to take into account the variation in the concentration of free ligate as well as that of free affinity sites [30,32]. This inability to assume constancy of [$\bar{A}$] gives rise to the differential rate equation.

$$d[AX]/dt = k_a[\overline{\overline{A}}][\overline{\overline{X}}] + k_a\{[AX]^2 - ([\overline{\overline{A}}] + [\overline{\overline{X}}])[AX]\} - k_d[AX] \quad (24)$$

in which $[\overline{\overline{A}}]$ is the concentration of ligate placed in the cuvette. Numerical integration is used to determine the best-fit description of the time dependence of [AX], which is obtained by applying Eq. (14) to the biosensor trace. The need to evaluate three curve-fitting parameters, k_a, k_d and $[\overline{\overline{X}}]$, may be obviated by prior thermodynamic characterization of the interaction (Section 4.3) to obtain values of $[\overline{\overline{X}}]$ and K_{AX}. On the basis of numerical integration of the differential equation expressed in the form

$$d[AX]/dt = k_a([\overline{\overline{A}}][\overline{\overline{X}}] + [AX]^2 - \{[\overline{\overline{A}}] + [\overline{\overline{X}}] + (1/K_{AX})\}[AX]) \quad (25)$$

the curve-fitting simplifies to evaluation of the single parameter, k_a. Further details of this second-order kinetic approach appear in studies of the interactions of carboxypeptidase A [30] and histidine-rich glycoprotein [32] with immobilized forms of their respective elicited antibodies.

In some of the above studies it has been possible to develop quantitative expressions that make allowance for the operation of the particular complicating factor being considered. However, the general problem of devising a kinetic analysis to accommodate the combination of potential complicating factors is seemingly intractable in the absence of information about the relative contribution of each phenomenon to the deviation from pseudo-first-order kinetic behaviour. We therefore conclude this section by describing a kinetic analysis that is more akin to the corresponding thermodynamic analysis (Section 4.1).

5.3. A kinetic equivalent of the thermodynamic approach

As noted earlier (Section 3.2), current analyses of biosensor traces are merely providing phenomenological rate constants pertaining to thermodynamic description of the interaction as a single-phase system. What is therefore required from the kinetic analysis of a given time dependence of biosensor response is the best-fit value of the pseudo-first-order rate constant (k_{pfo}) obtained on the basis of Eq. (18) with the magnitude of R_e fixed at its plateau (equilibrium) value. This approach is illustrated in Fig. 10a for the interaction of the Fab fragment of an anti-paraquat antibody with a BIAcore chip on which the carboxymethyldextran layer had been extensively derivatized with eliciting antigen [25]. Although such analysis provides a poor description of the experimental time dependence of biosensor response, its good description was not the object of the exercise. Indeed, an advantage of this treatment is its ability to accommodate a varying extent of deviation from pseudo-first-order kinetic behaviour in experiments with different ligate concentration – a problem that plagues analyses based on multiexponential curve-fitting [22,57,63–68]. The dependence of the resultant k_{pfo} values upon injected ligate concentration is presented in Fig. 10b, which yields estimates of $9.4(\pm 0.2)\times 10^3\ M^{-1}\ s^{-1}$ and $3.9(\pm 1.1)\times 10^{-3}\ s^{-1}$ for $(k_a)_{pfo}$ and $(k_d)_{pfo}$ from the slope and ordinate intercept respectively (Eq. (17b)). As noted in the original study [25], the estimate of $2.6(\pm 0.8)\times 10^6\ M^{-1}$ for K_{AX} that is obtained from their ratio essentially duplicates the value inferred from Fig. 9b for the same interaction under con-

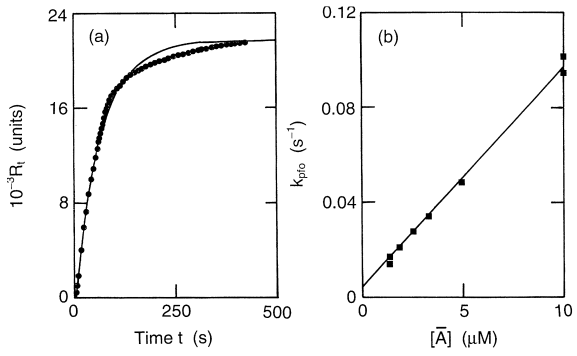

Fig. 10. Kinetic counterpart of the thermodynamic analysis of BIAcore results deviating from pseudo-first-order behaviour. (a) Adsorption stage of the progress curve obtained by passage of 1.25 μ*M* anti-paraquat Fab fragment over a BIAcore sensor chip heavily modified with immobilized antigen (●), together with the best-fit description (Eq. (18)) in terms of a pseudo-first-order rate constant, k_{pfo}, and the predetermined value of the equilibrium response, R_e. (b) Use of the dependence of the pseudo-first-order rate constant upon ligate concentration to evaluate the effective association and dissociation rate constants via Eq. (17b). Data taken from Ref. [25].

ditions (lower $[\overline{\overline{X}}]$) commensurate with pseudo-first-order kinetic behaviour – conditions where this procedure and the conventional kinetic analysis become synonymous.

A kinetic counterpart of the thermodynamic approach may seem redundant in the sense that its application requires specification of the equilibrium response at each $[\overline{A}]$, the analysis of which in terms of Eq. (2) should surely provide K_{AX} in its own right. However, such logic presupposes the availability of data for a range of ligate concentrations that allows accurate definition of the rectangular hyperbolic dependence of R_e upon $[\overline{A}]$. In fact, direct thermodynamic characterization was precluded in the above study, where all values of R_e reflected extents of matrix-site saturation greater than 90%.

This demonstrated ability of the kinetic analog of the thermodynamic approach to evaluate K_{AX} from sensor traces reflecting essential saturation of the affinity matrix is encouraging from the viewpoint of characterizing high-affinity interactions by BIAcore technology. In principle, the flow design of the BIAcore instrument has the potential to allow definition of the ligate concentration in the liquid phase, $[\overline{A}]$, provided that a sufficient volume of solution can be injected to establish the magnitude of the plateau response (R_e). Because [AX] needs to be in the nanomolar to micromolar range to meet instrumental sensitivity requirements, an extremely high level of sensor-surface derivatization with affinity sites may be necessary in order to obtain recordable sensor traces. Irrespective of the extent to which those time dependences conform with pseudo-first-order kinetic behaviour, they may be examined in such terms (Fig. 10) to obtain K_{AX} in the event that 1:1 stoichiometry for the interaction is a reasonable assumption.

6. Concluding remarks

A major feature of this review has been its focus on theoretical rather than experimental aspects of the characterization of interactions by biosensor technology – an approach adopted to emphasize the importance of extracting meaningful information from quantitative studies of interactions by biosensor technology, and to establish the significance of the parameters thereby obtained. Of particular note in that regard is the demonstration that the conventional kinetic analysis provides little information about the actual chemical mechanism of the interaction between ligate and affinity sites immobilized on the sensor surface. Instead, it is providing an alternative means of obtaining the thermodynamic description in terms of an equilibrium reaction confined to a single phase. Furthermore, that characterization refers to the interaction of ligate with a chemically modified (immobilized) form of its affinity partner; and does not, therefore, necessarily describe the corresponding biospecific interaction in solution. Characterization of the latter interaction requires the adoption of a form of competitive binding assay whereby the ligate interaction with affinity matrix sites is used to monitor its free concentration in a liquid phase comprising a mixture of ligate and the soluble (underivatized) form of its affinity partner [30,43,59,68,71,72]. In the sense that the need for the conduct of such competition experiments has always been central to the development of quantitative affinity chromatography for the characterization of interactions, it is surprising that so little attention has been directed towards this aspect of their study by biosensor technology.

This review has also served to place the characterization of interactions by biosensor technology in the broader context of their study by quantitative affinity chromatography. Because biosensor technology provides a further variant of methodology for the characterization of ligand binding by quantitative affinity chromatography, advantage should clearly be taken of the theory and experience gained from development of that well-established technique. Biosensor technology has the particular advantage of affording the opportunity for application of those quantitative expressions to interactions that are precluded from study by forms of quantitative affinity chromatography that monitor complex formation on the basis of the extent of ligate depletion in the liquid phase. In providing a means of monitoring complex formation directly, biosensor technology adds another dimension to the versatility of quantitative affinity chromatography by extending the range of measurable affinity constants to include interactions that were too strong for study by existing variants of the procedure.

At this stage the quantitative study of interactions

by biosensor technology is still in relative infancy: indeed, the optimization of methodological and analytical details is almost certainly incomplete. Many of the quantitative studies have been designed in the belief that the major contribution of biosensor technology would be its provision of information on kinetics and mechanism – the reason for the expenditure of a great deal of experimental and theoretical effort to refinement of the kinetic analyses. However, it is now evident that a more important attribute of the technique is likely to be its potential for thermodynamic characterization of interactions that are not amenable to study by other means. In that regard an extremely exciting prospect is the use of suitably modified sensor surfaces to mimic the membrane surface for the quantitative characterization of interactions involving membrane-localized receptors [73–79]. For these interactions the affinity chromatography system is not merely being used as a means for approaching the problem of characterizing a corresponding reaction in the liquid phase: the biospecific interaction of interest is, indeed, the uptake of ligate from the liquid phase. Such studies show every promise of extending considerably a potential that has already been exploited in relation to the use of quantitative affinity chromatography for the characterization of interactions between glycolytic enzymes and the myofibrillar matrix [9,80–83]. We hope that this review may stimulate further interest in the deployment of quantitative affinity chromatography, particularly the biotechnology variant thereof, for the characterization of the vast array of biospecific interactions thereby rendered amenable to quantitative study.

7. Glossary of symbols

A	partitioning solute
$[\underline{A}]$	free ligate concentration
$[\underline{\underline{A}}]$	total ligate concentration
$[\overline{A}]$	total ligate concentration in the liquid phase
$[A]_s$	free ligate concentration in the stationary phase
$[\overline{A}]_s$	total ligate concentration in the stationary phase
f	valence of ligate
F	biosensor proportionality constant
k_a	association rate constant for ligate–matrix site interaction
k_d	dissociation rate constant for ligate–matrix site interaction
k_{obs}	measured pseudo-first-order rate constant
K_{AS}	equilibrium constant for ligate–competitor interaction
K_{AX}	equilibrium constant for ligate–matrix site interaction
$\overline{K}_{AX}$	constitutive equilibrium constant in presence of competitor
$(K_{AX})_s$	equilibrium constant in terms of stationary phase concentrations
M_A	molecular weight of ligate
Q	ratio of ligate–matrix site equilibrium constants $(K_{AX}/\overline{K}_{AX})$
R	biosensor response
R_e	biosensor response at equilibrium
R_m	maximal biosensor response
R_t	biosensor response at time t
S	soluble ligand competitive with matrix sites for ligate
$[\underline{S}]$	free concentration of competing ligand
$\underline{\underline{S}}$	total concentration of competing ligand
V	volume accessible to ligate
X	affinity site on matrix
$[\underline{X}]$	free concentration of matrix sites
$[\underline{\underline{X}}]$	total concentration of matrix sites in volume V
$[\overline{X}]_s$	total matrix site concentration in stationary phase
ϕ	fractional volume comprising stationary phase
σ_A	partition coefficient of ligate

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