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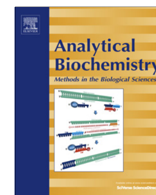

An analytical solution to the characterization of antigen–antibody interactions by kinetic exclusion assay

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ABSTRACT

A simpler derivation of the basic expression for the dependence of fluorimetric response ratio (R_{Ag}/R_o) on free antigen concentration has demonstrated the universal invalidity of the analysis that is incorporated into the manufacturer's software for determining immunoaffinities by kinetic exclusion assay, and traced the error to inadequate allowance for antibody bivalence in the solution phase of the assay. An analytical solution to the quantitative characterization of antigen–antibody interactions from the dependence of R_{Ag}/R_o on total antigen concentration is also described, thereby eliminating the necessity for the extensive simulative procedures employed in current determinations of dissociation constants by kinetic exclusion assay. In the illustrative application of this analytical approach to published results on the interaction between a metal chelate (cadmium–ethylenediaminetetraacetic acid, Cd–EDTA) and an elicited monoclonal antibody, the analytical processing of the data has been performed on a calculator. However, there is no need to replace the more sophisticated procedure that is incorporated into the Sapidyne software provided that programming changes are made to rectify the erroneous equation on which the simulative analysis is based.

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Of the various methods that are currently employed to screen the affinities of immunochemical reactions in the search for therapeutic antibodies, the procedure with greatest potential for accurate quantification of an antigen–antibody interaction is the kinetic exclusion assay [1–7], the throughput of which can be enhanced substantially by its conduct on a Gyrolab workstation [8]. The superiority of this KinExA[1] approach resides in its characterization of the reaction between native forms of the antigen and antibody in a homogeneous solution. On the other hand, the more popular technique based on surface plasmon resonance [9–11] characterizes the biphasic interaction between antigen and a chemically modified (immobilized) form of the antibody [12–15]. Although the biologically relevant interaction can be characterized by extending the experiment to examine the effect of including antibody as well as antigen in the solution flowing across the biosensor chip [12–15], that improvement in experimental design comes at the expense of screening throughput.

The kinetic exclusion assay is a biphasic immunoassay that monitors the concentration of free antibody sites in a reaction mixture with defined total concentrations of antibody and antigen that has attained chemical equilibrium prior to analysis. After exposure of this equilibrium mixture to an affinity matrix with a high concentration of immobilized antigen to capture molecules with free antibody sites, the amount of captured antibody is monitored fluorimetrically by adding a labeled goat anti-human immunoglobulin G (IgG) as the KinExA detection antibody. The concentration of free antibody in the mixture is quantified as the ratio of the fluorescence response for the mixture (R_{Ag}) to that (R_o) for the same total antibody concentration in the absence of antigen, for which expressions are available only in terms of the free antigen concentration in the reaction mixture [5,6,15]: thus, the free antigen concentration is a parameter that also requires evaluation by the analysis. Whereas simulative [5,6] or iterative [15] approaches have previously been used to obtain the dissociation constant and free antigen concentration as best-fit parameters describing the dependence of $(1 - R_{Ag}/R_o)$ on total antigen concentration in equilibrium mixtures with a fixed total antibody concentration, an analytical solution to the problem has now been devised.

Although the KinExA procedure has the potential to provide accurate measurements of affinity constants, a worrying aspect of the current computer program for so doing [5,6] concerns the adequacy of the allowance for antibody (IgG) bivalence. Basically, it is assumed that doubling of the antibody concentration allows analysis of the antigen–antibody interaction in terms of theory developed for 1:1 binding. In that regard, a previous theoretical investigation [15] had seemingly identified conditions under which that assumption would be a justifiable approximation, but it transpires that those conditions are incompatible with the design

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[1] Abbreviations used: KinExA, kinetic exclusion assay; IgG, immunoglobulin G; Cd–EDTA, cadmium–ethylenediaminetetraacetic acid; DPTA, diethylenetriaminepentaacetic acid; ELISA, enzyme-linked immunosorbent assay.

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of a KinExA experiment. The results of subsequent deliberations, including the above-mentioned analytical determination of the free antigen concentration and dissociation constant, form the basis of this investigation.

Theoretical considerations

As noted above, the kinetic exclusion assay monitors the concentration of free antibody in a reaction mixture with defined total concentrations of antigen and antibody ($[Ag]_{tot}$ and $[Ab]_{tot}$, respectively) that has attained chemical equilibrium prior to analysis. This equilibrium mixture is then reacted with a chromatographic matrix on which the concentration of affinity sites (immobilized antigen Ag^*) on the poly(methyl methacrylate) beads is sufficiently high relative to antibody concentration ($[Ag^*]_{tot} \gg 2[Ab]_{tot}$) to ensure essential constancy of free antigen concentration in the matrix phase ($[Ag^*]$). On the grounds that the extremely small rate constant governing dissociation of antigen–antibody complexes should ensure that essentially no discernible reequilibration accompanies the consequent removal of antibody molecules from the solution phase, the fluorescence response of the gel phase (R_{Ag}) reflecting removed antibody is reflecting the combined concentrations of free antibody (–Ab–) and the two forms of complex (–Ab–Ag and Ag–Ab–) with one site still available for interaction with immobilized antigen (Ag^*). Specifically, the rate of removal of antibody from the solution phase is described by the expression

$$d[Ab]/dt = -[Ag^*]\{2k[-Ab-] + k([-Ab-Ag] + [Ag-Ab-])\}, \tag{1}$$

where, because of the essential constancy of $[Ag^*]$, the product of free immobilized antigen concentration and k (the second-order rate constant for interaction between free Ag^* and free antibody sites) becomes a pseudo-first-order rate constant. Although the probability of free antibody removal is twice that for the removal of antigen–antibody complex, this seeming preference for free antibody uptake by the affinity matrix is countered by the fact that the concentration of the latter includes equal contributions from two antigen–antibody complexes. The fluorescence response of the affinity gel, thus, provides a reliable index of the concentration of free antibody sites removed from the solution phase irrespective of their existence on unreacted antibody or on singly linked antigen–antibody complexes. It should be noted that the above reasoning is conditional on equivalence and independence of the two antibody sites in their interaction with immobilized antigen but makes no assumption about the relative magnitudes of the dissociation constants for the immunochemical reactions in the gel and solution phases. Furthermore, the extremely small dissociation rate constant for an interaction with an intrinsic dissociation constant in the nanomolar–picomolar range justifies the approximation that no dissociation of complex(es) occurs as the result of antibody removal during the few seconds in which mixture is exposed to the gel phase [4,16].

On the grounds that the only solution species failing to interact with the affinity matrix is the complex Ag–Ab–Ag, ($1 - R_{Ag}/R_o$) is monitoring its concentration as a proportion of the total antibody concentration. Incorporation of the concept of equivalent and independent interaction between antigen and antibody sites [17] leads to the expression

$$\left[1 - (R_{Ag}/R_o)\right] = \frac{[Ab]\left([Ag]_e/K_d\right)^2}{[Ab]\{1 + \left([Ag]_e/K_d\right)\}} = \frac{\left([Ag]_e/K_d\right)^2}{\{1 + \left([Ag]_e/K_d\right)\}^2}, \tag{2}$$

where K_d is the intrinsic dissociation constant. To achieve a rectangular hyperbolic dependence on $[Ag]_e$, the square root of Eq. (2) needs to be taken, after which the relationship

$$\left[1 - (R_{Ag}/R_o)\right]^{1/2} = \frac{[Ag]_e/K_d}{1 + \left([Ag]_e/K_d\right)} = \frac{[Ag]_e}{K_d + [Ag]_e} \tag{3}$$

is obtained. This expression now needs to be rationalized with the earlier findings [15].

By means of reacted site probability theory [18,19], it was shown previously [15] that the fluorescence response ratio is given (Eq. (8) of Ref. [15]) by

$$\frac{R_{Ag}}{R_o} = \frac{(2 - P_S) + 2[Ag]_e/K_d}{(2 - P_S)\left(1 + [Ag]_e/K_d\right)^2}, \tag{4}$$

where P_S denotes the probability that an unoccupied antibody site has reacted with immobilized antigen Ag^*. This expression simplifies to Eq. (3) on setting P_S equal to unity (see Eq. (11) of Ref. [16])—the situation signifying the interaction of all free antibody sites with the affinity matrix if the biphasic interaction were allowed to proceed to its final state. In retrospect, unity is the only allowable value for P_S because the high concentration of immobilized antigen used to justify the approximation that $[Ag^*]$ remains essentially constant also ensures the capture of essentially all free antibody sites on attainment of the biphasic equilibrium state. Any other P_S value would signify nonfulfillment of that basic requirement for validity of the KinExA protocol.

The first form of Eq. (3) is readily rearranged to yield

$$\frac{[Ag]_e}{K_d} = \frac{\left[1 - (R_{Ag}R_o)\right]^{1/2}}{1 - \left[1 - (R_{Ag}R_o)\right]^{1/2}} \tag{5}$$

as one expression for $[Ag]_e/K_d$. Furthermore, definition of the intrinsic dissociation constant for the solution phase interaction in terms of the relevant ratio of species equilibrium concentrations as

$$K_d = \frac{[Ag]_e(2[Ab]_{tot} - [Ag]_{tot} + [Ag]_e)}{([Ag]_{tot} - [Ag]_e)} \tag{6}$$

leads to a second expression for $[Ag]_e/K_d$, namely,

$$\frac{[Ag]_e}{K_d} = \frac{[Ag]_{tot} - [Ag]_e}{2[Ab]_{tot} - [Ag]_{tot} + [Ag]_e} \tag{7}$$

which on rearrangement yields the following expression for $[Ag]_e$:

$$[Ag]_e = \frac{[Ag]_{tot} - \left([Ag]_e/K_d\right)\left(2[Ab]_{tot} - [Ag]_{tot}\right)}{\left([Ag]_e/K_d\right) + 1}. \tag{8}$$

Substitution of the value of (Ag/K_d) deduced from Eq. (5) into this expression yields an unequivocal estimate of $[Ag]_e$, after which the magnitude of the dissociation constant then follows as the ratio of $[Ag]_e$ to $[Ag]_e/K_d$.

Alternatively, the value of $[Ag]_e$ can be combined with the total concentrations of antibody and antigen ($[Ab]_{tot}$ and $[Ag]_{tot}$) to obtain the fractional saturation of antibody sites in the equilibrium mixture (F), and hence K_d via the expression

$$F = \frac{[Ag]_{tot} - [Ag]_e}{2[Ab]_{tot}} = \frac{[Ag]_e}{K_d + [Ag]_e}, \tag{9}$$

which describes the rectangular hyperbolic dependence of F on free antigen concentration.

The above prediction of a linear dependence of $[Ag]_e$ on $[Ag]_e/K_d$ (or of a rectangular hyperbolic dependence of F on $[Ag]_e$) is, of course, conditional on the validity of assigning the same dissociation constant to all interactions between antigen and antibody sites in the equilibrium mixture. As in all ligand binding studies, departure from this simplest situation involving equivalence and independence of antibody sites in their interactions with antigen [17] is recognized by nonconformity of an experimental data set with those predictions. Besides affording a quantitative characterization of immunochemical reactions for which assumed equivalence and independence of the two IgG sites is a reasonable approximation, the current analysis also has the potential to identify situations in

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