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An Overview of ELISA-Based Initial Velocity Methods to Measure the Immunoreactive Fraction, Association Rate, And Equilibrium Constants of Monoclonal Antibodies

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ABSTRACT

Three biochemical parameters—the on-rate (k_{+1}), the dissociation rate (k_{-1}), and the equilibrium (K_a) constants—describe the antibody interaction with a ligand. In addition, the antibody immune reactive fraction (IRF) is important because it affects k_{+1} and K_a constant values. The on-rate and dissociation constants are kinetic, but the equilibrium constant is a thermodynamic parameter, and the methods for their determination are different. Among the available methods to determine these parameters, only surface plasmon resonance (SPR)-based techniques allow calculation of the three antibody constants in a single experiment. However, conventional immunoassays are also suitable to determine antibody constants. Here, we describe some enzyme immunoassays (ELISA) methods developed in our laboratory to, i) quantitate mAb in culture supernatants from the initial velocity of the progress curve, ii) determine mAb IRF by a two-step ELISA method, iii) calculate mAb on-rate constant by kinetic ELISA based on time-course data of ligand binding to capture mAb, and, iv) describe a competitive-inhibition ELISA to measure the inhibition constant (K_i) of mAb binding to plate-bound ligand, in the presence of competing concentrations of mAb and soluble ligand.

Abbreviations: mAb, monoclonal antibody; [ ], means concentration; HRP, horse radish peroxidase; kobs, observed on-rate constant; Ks, Michaelis dissociation constant; Vmax, limiting velocity; NLR, non-linear regression

INTRODUCTION

Immunoglobulins are plasma proteins secreted by B-lymphocytes which constitute a main protective component of innate and adaptive humoral immune systems. Immunoglobulins are characterized by a series of physicochemical properties including heterogeneity, specificity, affinity, and polyreactivity. For many years, the heterogeneous composition of immunoglobulins hampered the characterization of biochemical parameters which are essential for predicting and assessing their biological performance. In 1975, George Köhler and Cesar Milstein produced immortalized cells that secreted monoclonal antibodies [1]. With the introduction of mAb technology came the vast development of mAb-based methods that are now widely used in biological research, clinical diagnosis [2], cancer immunotherapy [3], and autoimmune and inflammatory illnesses [4]

Proper analysis of mAb-ligand interaction requires knowledge of antibody concentration, its functional (immunoreactive) fraction (IRF), and three biochemical parameters: the association (k_{+1}) and dissociation (k_{-1}) rate constants, and the equilibrium constant ($K_a = 1/K_i$). Some of these parameters are interdependent; the on-rate and equilibrium constants depend on the IRF value which determines mAb functional concentration. Immunoassay sensitivity is subordinated to the affinity constant (K_a). The dissociation rate constant (k_{-1}) specifies ligand residence time ($1/k_{-1}$) and the half-life ($\log_e 2/k_{-1}$) of its interaction with the antibody, and the association rate constant (k_{+1}) relates the fraction of reactant molecules bound per unit of time to their concentrations, and signalize

the velocity of antigen detection and quantification [5, 6]. Knowledge of mAb kinetic (k_{+1} and k_{-1}) and equilibrium (K_a) constants is thus crucial for predicting antibody performance.

Nowadays for routine analyses high throughput data acquisition based on surface plasmon resonance (SPR) offers rapid return of antibody kinetic and equilibrium data; this way of doing, nevertheless, has become a custom-based analysis in which the researcher has a limited role. In laboratories which lack expensive instrumentation, a convenient alternative to SPR technology is to use low-cost methods like ELISA which are simple, sensitive and specific. ELISA and SPR measure biphasic (heterogeneous) interactions based on different technologies; nonetheless, mAb kinetic and equilibrium constants derived by both methods show close similarity [7, 8].

The study of enzymes was at the heart of Biochemistry for more than thirty years in which enzymologists lent to biology a wealth of plots and methods of analysis with which to quantitate molecular interactions. Antibody-ligand binding follows the same molecular recognition mechanisms as those of an enzyme and its substrate. On that basis, we used enzyme kinetic (k-ELISA) principles to study the interaction between mAb and ligand [9]. ELISA is a simple, low-cost technology that can be developed in a range of creative formats to implement sensitive analytical tools that can be adapted to a variety of detection systems.

Three excellent textbooks that show the principles of enzyme kinetics and how they apply to mAb analysis are [10-13]. Here, we provide a brief account of ELISA-based kinetic methods developed in our laboratory. The references in each section marked with an asterisk (*) provide a detailed explanation of the experimental methods, and more in-depth information of the relevant literature.

QUANTITATION OF MAB CONCENTRATION IN CULTURE SUPERNATANTS

In order to quantify the amount of mAb in culture supernatants, we described a non-competitive two-site ELISA that is based on the principle that the gradient of rate measurements within the linear range of the initial velocity is proportional to analyte concentration [14*]. To capture the mAb in the test sample plate wells were coated with a species-specific anti-mouse globulin; subsequently, bound mAb was reacted with anti-mouse immunoglobulin conjugated to reporter enzyme (horseradish peroxidase; HRP), and the reaction velocity recorded within the first minute of incubation. All reaction components: anti-mouse globulin, indicator reagent (anti-mouse-HRP) and the enzyme substrate (ortho-phenylenediamine; OPD) must be present in molar excess relative to the analyte (mAb) concentration [9]. ELISA quantitation is usually done by end-point measurement, and the reaction product recorded by a single readout after extended incubation (> 10 min). These conditions, however, do not ensure readout linearity. We recommend to make sample measurements within the initial phase of the progress curve when the enzyme catalysis proceeds in steady-state conditions and the slope of the curve is proportional to the amount of ligand measured.

Antibody quantitation is based on comparison of a mAb sample of unknown concentration to a calibration curve. The linear range of the curve is established with an IgG standard usually made with purified antibody of polyclonal or monoclonal origin. Polyclonal IgG standards express different number and heterogeneity of epitopes which recognized by the indicator reagent (anti-mouse-HRP) can generate calibration curves with distinct slopes and produce discrepant results. A better IgG standard should be based on a mAb of the same isotype as the analyte being measured.

To build the calibration curve a dilution series of IgG standard (from 0 to 8 ng/ ml) in triplicate, were added to anti-mouse globulin-coated wells. The anti-mouse globulin-IgG complexes were then incubated with a molar excess of anti-mouse-HRP, and bound IgG measured at fixed time intervals for the first 120 s of the reaction. The slope of the captured IgG begins to lose linearity after 60s of incubation, time which was chosen to register enzyme activity. Nevertheless, it advisable to measure reaction rate at a shorter incubation time (30–40 s), as proportionality between product formation and analyte (mAb) concentration was better maintained.

To quantitate mAb in samples an IgG standard concentration series, by triplicate, was plated in parallel with a seven-dilutions series of culture supernatant, for instance 1/17,000, 1/15,000, 1/13,000, 1/11,000, 1/9,000, 1/7,000 and 1/5,000. Relative concentrations were calculated: 1/17,000 = 1, 1/15,000 = (17: 15) = 1.13, 1/13,000 = (17: 13) = 1.31, 1/11,000 = (17: 11) = 1.54, 1/9,000 = (17: 9) = 1.89, 1/7,000 = (17: 7) = 2.43, 1/5,000 = (17: 5) = 3.4. The plate was incubated (16 h, 4º C), washed three times with PBST (PBS with 0.05% Tween-20), and the indicator reagent (anti-mouse-HRP) added. After incubation (1h, 37 ºC) the plate was washed three times with PBST and the enzyme reaction initiated by adding HRP substrate. After 60s, the reaction was terminated and the product measured by absorbance at $A_{492\ nm}$. IgG bound (mAb captured/min) was plotted against the relative concentration for diluted mAb samples. mAb concentration in culture supernatants were determined by interpolating bound IgG values ($A_{492\ nm}$) (Figure 1B) from an IgG calibration curve (Figure 1A) with GraFit 7 software.

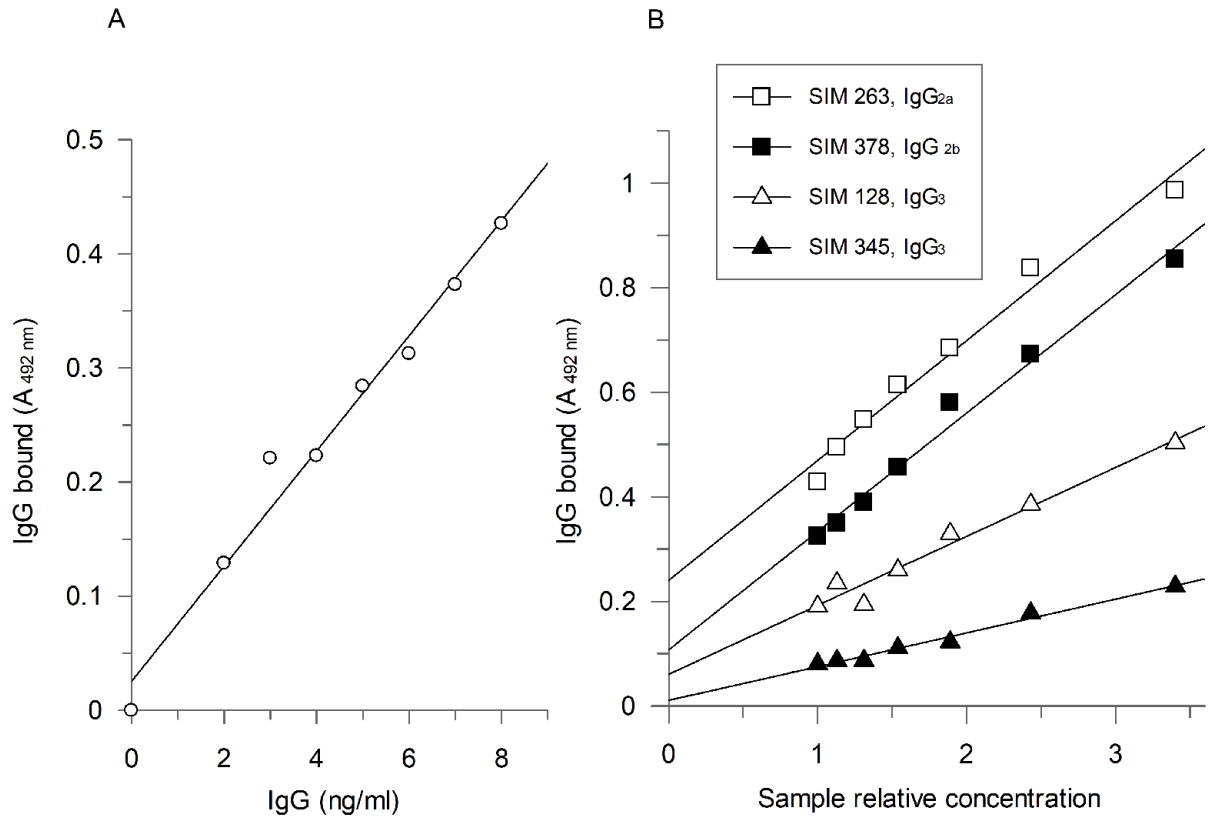

Figure 1: mAb quantitation based on IgG polyclonal-generated calibration curve. A. IgG calibration curve made with a mouse polyclonal IgG. IgG bound (y-axis) against IgG standard (ng/ml) (x-axis). B. Quantitation of four mAb samples. IgG-bound (mAb captured/min) (y-axis) against the relative concentration of diluted series of mAb samples (x-axis).

DETERMINATION OF MAB IMMUNOREACTIVE FRACTION

We have not found mAb culture supernatants which IRF values close to 100%, which suggests that a sizable portion of the molecules synthesized and secreted by hybridoma cells are non-reactive. In addition, during purification, labeling, or conjugation to other molecules antibodies lose some of their functionality. The remaining activity is called antibody immunoreactive fraction (IRF). Determination of antibody IRF is an important and necessary quality control for immunoglobulin-based reagents. Inactive molecules in antibody preparations can introduce error in the calculation of antibody constants, making it necessary to estimate the IRF before determining the K_a. The detrimental effect of non-functional molecules on K_a is more evident for high affinity antibodies. In these cases, the free antibody concentration at equilibrium is very small, and the use of uncorrected antibody concentration would introduce large false free antibody values in the linear transformed plots [15].

Binding data analysis and linearly converted graphs of the mass action law can be used to compute antibody IRF [16, 17]. The most popular of these techniques is a linear variant of the Lineweaver-Burk plot [18]. In this cellular assay, free and cell-bound antibody fractions at equilibrium are plotted as the percentage of [total]/[bound] antibody (on the y-axis) against the inverse of cell antigen concentration, 1/[cells] (on the x-axis). At hypothetical infinite antigen concentration, 1/[cells] = 0, and the extrapolated line intersects the y-axis at y = 1/r, where r is the inverse of the IRF value. The reliability of IRF values depends in part on the asumptions that total and free antigen concentrations are comparable at high antigen excess and that all functional antibody molecules are bound to antigen. These requirements are only met when [cell epitopes] > > $1/K_a$ and antibodies have high affinity). In analyses using cells with low antigen expression, however, or with low affinity mAb (K_a ~10^7 –10^8 M^{-1}), the Lindmo method can generate less accurate IRF data [19].

To improve and facilitate antibody IRF determination, we developed a two-step ELISA in antigen excess conditions [20*]. Two 96-well plates were used for each IRF determination. The

method involves titration of a mAb at limiting dilution in a first plate against a series of increasing plate-bound antigen concentrations until mAb binding reaches a plateau. At this point, all functional mAb molecules should be antigen bound except in wells in which antibody titer is higher than antigen concentration. Supernatant aliquots containing free mAb are transferred to a second plate coated with antiglobulin, and incubated with anti-mouse globulin-HRP to determine mAb-bound fractions. The time course of the enzyme reaction was measured during the linear phase period at 0, 10, 20 and 40 s of incubation. In excess of HRP substrate, the amount of antiglobulin-bound mAb at each antigen concentration is directly proportional to the initial velocity gradients (G_i), which were obtained by linear fit. mAb IRF was determined by computing the ratio (G_i/G_o) between the velocity gradient (G_i) of each reaction and the gradient of the reaction in the absence of antigen (G_o) (total mAb). The fraction of antigen-bound mAb is given by 1- G_i/G_o, and the percent IRF was calculated for each antigen concentration as % IRF = 100 (1- G_i/G_o). % IRF progress curves from four mAb determinations are shown [20*]. In addition, the SIM 169-3 anti-bovine serum albumin (BSA) % IRF progress curve is shown (Figure 2).

IRF determination by kinetic ELISA is not constrained by equilibrium conditions posed by the Lindmo assay, does not require knowledge of antigen concentration, and can be used to determine the IRF of poly- and monoclonal antibodies. The antigen concentration range for plate coating is determined by the adsorption conditions (concentration, incubation time, and temperature) prior to experimental use. Likewise, mAb dilutions for IRF assessment can be established by checkerboard analysis by titrating a series of mAb dilutions against a range of plate-adsorbed antigen concentrations. Kinetic measurement of the initial velocity of an enzyme reaction is very sensitive method, simple, inexpensive and highly repeatable.

DETERMINATION OF MAB ON-RATE (K_{+1}) CONSTANT

The majority of physiological events take place in non-equilibrium systems [21], hence when assessing the biological activities of an antibody, its kinetic properties should also be taken into account. According to research on the *in vitro* neutralization of various virus families, the antibody k_{+1} value is connected to stronger neutralization potency (lower antibody concentration) [22], and it is a better predictor of host protection than antibody affinity [23-25].

We report a kinetic ELISA to help with the quantification of mAb on-rate constants based on time-course data of ligand (L) binding to plate-bound mAb at various starting [L]. This reaction follows second order kinetics with a rate equation, $v = k_{+1}$ [mAb] [L]. To simplify the analysis, the ELISA was done under *pseudo-first* order conditions ([L] > > [mAb]).

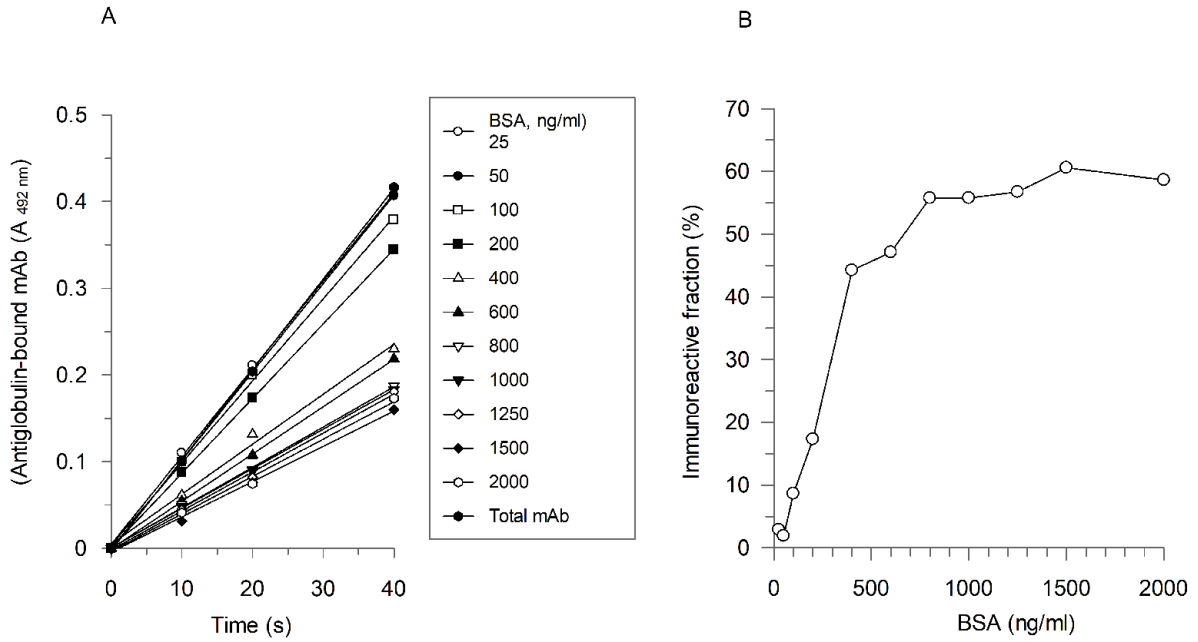

Figure 2: Progress curve of % IRF values of mAb SIM 169-3 anti-BSA. A. Initial velocity plot (0 to 40 s) of free mAb fractions from which gradient values (G_i) for each antigen concentration were obtained by linear fit. B. Progress curve of mAb % IRF as a function of BSA coating concentrations.

This reaction follows first order kinetics with equation v = k' [mAb], in which k' (= k_{obs}) is a *pseudo-first* order rate constant. The second order rate constant (k_{+1}) is calculated from $k_{+1} = k_{obs}/ [L]$. The method requires knowledge of antigen and antibody concentrations to establish the [antigen]/[mAb] ratios used, and a ligand conjugation reaction with biotin.

The ELISA below describes the interaction between mAb SIM 253-19 (SIM 253) and cholera toxin (CT) [26*]. The time-course data are the mean values of readings taken from two identical 96-well plates developed in parallel. Plates coated with anti-mouse globulin were incubated (30 min, 37 ºC) with a predetermined dilution of mAb SIM 253. After plate washing, 25 µl of PBST were added to all wells. Wells of rows A to H received 25 µl of an eight concentration series of twice-concentrated CT-biotin. The reaction was initiated by addition of 25 µl of the CT-biotin concentration series to wells of column 12 (time 0). At fixed times, 25 µl of CT-biotin concentrations were added to wells of columns 11 (2 min), 10 (3 min), 9 (4 min), 8 (5 min), 7 (6 min), 6 (6. 5 min), 5 (7 min), 4 (7.25 min), 3 (7.5 min), 2 (7.75 min) and 1 (8 min). The reaction was terminated by immersing the plates in PBST and draining between immersions. Streptavidin-peroxidase was added and incubated (1 h, 37 ºC). The reaction was developed (1 min) and the product measured by absorbance ($A_{492\ nm}$).

SIM 253-CT-biotin binding follows an exponential time-course described by a first-order rate equation, $P_t = P_m (1- e^{-k' t})$, where P_t is the amount of ligand bound at different time points and P_m the maximum binding; k' is a pseudo-first order constant (k_{obs}) with dimensions s^{-1}. The time-course initial velocity was analyzed by seven methods to derive k_{obs}, and, subsequently, k_{+1} by plotting k_{obs} against [CT-biotin]. Time-course data were fitted by nonlinear regression (NLR) to a first-order rate equation to obtain k_{obs}, and to Michaelis-Menten equation to derive the value of V_{max}/K_s, the vertical tangent of the curve. Additional methods to calculate k_{obs} were based on, a) the reaction half-time ($t_{1/2}$), which corresponds to K_s in the time-course curve, from which $k_{obs} = \log_e 2/K_s$. b) measurement of the initial velocity by Boeker's extrapolated-v_0 method [27], c) modified Hanes-Woolf and Lineweaver-Burk linearization plots, d) LOS plot [28], and e) linear plot of the initial rate gradient.

k_{obs} calculation from an exponential first-order rate equation, and from the V_{max}/K_s ratio with Michaelis relationship are quick and reliable procedures. Determination of k_{obs} from $\log_e 2/K_s$ generates a gradient slightly different from V_{max}/K_s. Nevertheless, taking the mean of both values yields a k_{+1} nearer the average. Boeker's approach yields reliable plots but is somewhat cumbersome due to the number of plots needed to calculate k_{obs}. Hanes-Woof and Lineweaver-Burk linearizations use reciprocals which makes them more liable to experimental error distortion. LOS data generated dispersed plots. Determination of on-rate constants from the initial velocity gradient must ensure that the ligand-bound fraction is within 5% and 10% of maximum binding; higher values imply less velocity and lower k_{obs} values.

To illustrate k_{obs} calculation, time-course data of mAb SIM 28-HRP interaction was fitted by NLR to a first-order rate equation (Figure 3) and to Michaelis equation (Figure 4). SIM 28-HRP binding time-course yielded a family of curves with different k_{obs} values as a function of HRP concentration (inset) (Figure 3A). The second-order rate constant k_{+1} was obtained from the slope of a k_{obs} against [HRP] plot (Figure 3B)

The k_{+1} for SIM 28-HRP binding was also determined using the V_{max}/K_s ratio, which shows the reaction rate at the very early reaction time. V_{max} and K_s parameters were derived from the time-course data by nonlinear regression (NLR) analysis adjusted to the Michaelis equation. The family of curves at varied [HRP] values, was comparable but not identical to that obtained by first-order rate analysis (Figure 4 A). The slope of a V_{max}/K_s plot against [HRP], yielded the second-order rate constant k_{+1} (Figure 4B)

In this experiment, the k_{+1} value ($2 \times 10^5\ M^{-1}\ s^{-1}$) generated by both methods is identical; nevertheless, the same data examined by various procedures can produce a value dispersion for k_{+1}, since the evaluation procedure may distort experimental mistakes to varying degrees. In these circumstances, it is more sensible to calculate an average k_{+1} by computing mean 95% confidence limits than expressing k_{+1} as a genuine number.

MEASUREMENT OF MAB DISSOCIATION CONSTANT (K_I) BY COMPETITIVE INHIBITION ELISA

The strength of the binding between an antibody and its ligand (affinity) is thought to be the best criterion for evaluating antibody activity, despite the fact that the majority of physiological events do take place in non-equilibrium systems [21]. The current gold standard method for measuring antibody affinity is SPR analysis, while ELISA methods are also frequently employed. In heterogeneous phase systems like SPR and ELISA, the interaction between a bivalent antibody and its antigen does not adhere to thermodynamic theory [29, 30], and affinity data from solid-phase tests is viewed as a measure of relative affinity [31]. Despite these drawbacks, solid-phase immunoassays have remained popular for determining antibody affinities, and several ELISA techniques are worthy of mention [32-35].

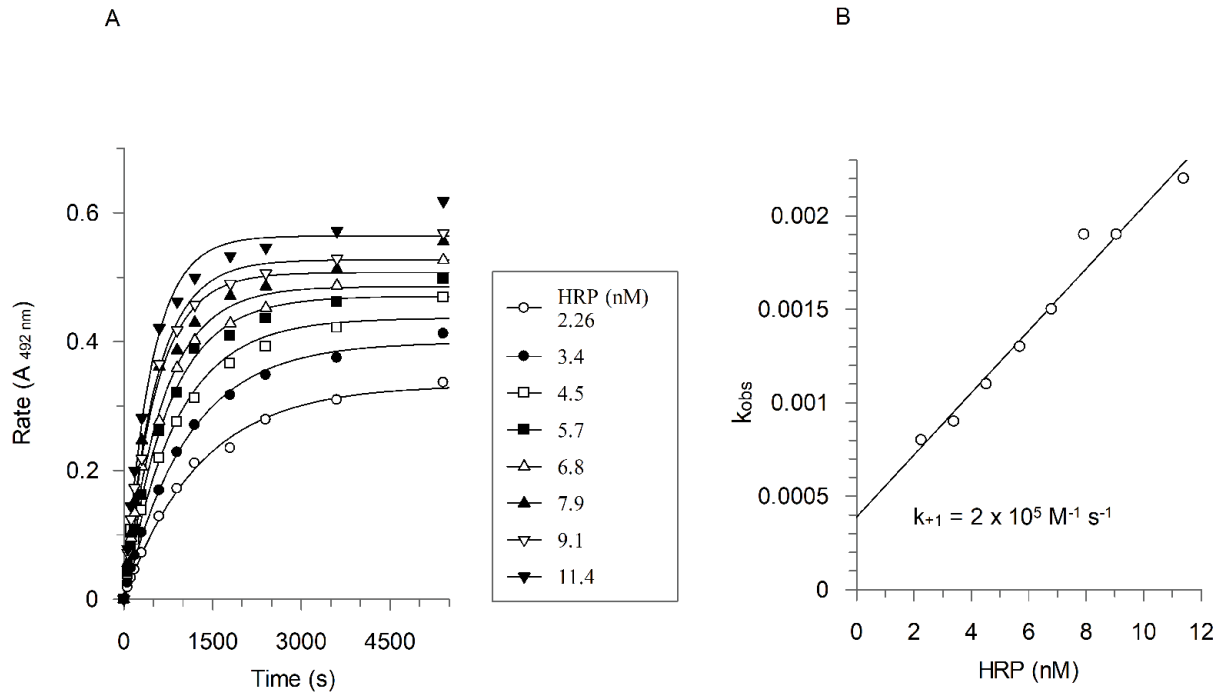

Figure 3: Analysis of SIM 28-HRP binding time-course data by first-order rate equation. A. mAb SIM28 was incubated for 0, 1, 2, 3, 5, 10, 15, 20, 30,40, 60 and 90 min with different HRP concentrations (inset). After incubation, the plate was processed as described and the initial rate (1 min) recorded ($A_{492\ nm}$). B. Calculation of the second-order rate constant from the gradient of a k_{obs} against [HRP] plot.

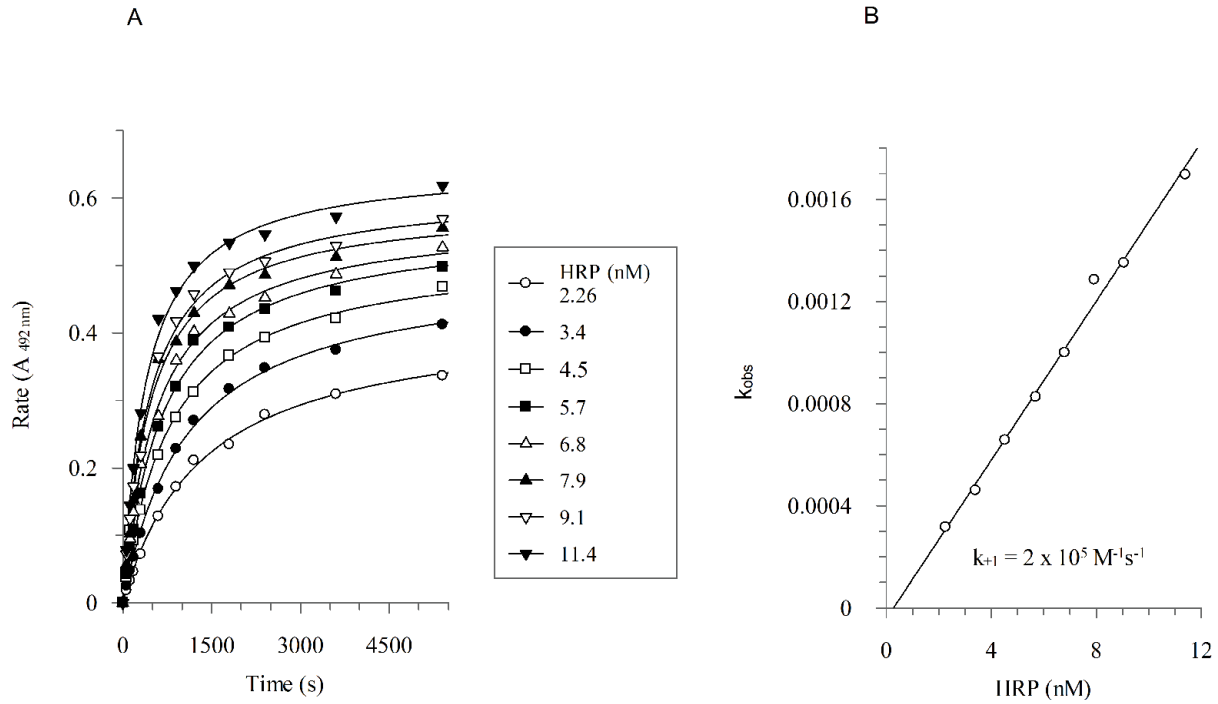

Figure 4: Determination of the initial rate of SIM 28-HRP binding time-course with Michaelis-Menten equation. A. Binding time-course curves as a function of [HRP] (inset). B. The second-order rate constant (k_{+1}) was obtained from the slope of plotting k_{obs} against [HRP]. Incubation times are as in Figure 3A.

To analyze mAb-antigen interaction we describe a competitive-inhibition ELISA based on the initial rate of mAb binding to antigen-coated wells (receptor), incubated with competing concentrations of the mAb (ligand; L) and the soluble antigen (inhibitor; I) [8*]. This competitive inhibition ELISA measures the mAb concentration bound to the immobilized antigen (receptor). The assay is set such that receptor epitopes > [L], and [I] >> [L]. In addition, it lacks the constraints of monitoring free antibody at equilibrium, does not require labeling or knowledge of antibody concentration, and inactive antibody molecules do not affect K_d measurements [15].

Based on the similarity of enzyme-substrate and mAb-inhibitor mechanisms, we applied enzyme linear competitive inhibition methods and the Michaelis rapid equilibrium approximation to study the inhibition constant (K_i) (moles/L) of mAb-inhibitor complex. Serially diluted samples of [mAb] and [inhibitor] were incubated to equilibrium. Plates were washed, and bound mAb measured with antiglobulin-peroxidase. The initial velocity data of receptor-bound mAb at various [mAb] and [inhibitor] were analyzed by NLR, linear transformations (Cornish-Bowden, Lineweaver-Burk, Hanes-Woolf, Dixon, Cortés [$1/i_{0.5}$ vs. V_i/V_{max}], Ascenzi [$K_s/V_{max}/K_{s,0}/V_{max}$ vs. [I]]) and NLR IC_{50} plots, to derive mAb inhibition constants (K_i).

The first step is to identify the mode of inhibition. This is done by plotting [mAb]/v against [I]. In linear competitive inhibition this plot yields a pattern of parallel lines whose intercept on the [mAb]/v axis increases as [mAb] increases, which provides unambiguous assignment of the inhibition type [36]. Once the inhibition mode is determined, K_s, V_{max}, and K_s/V_{max} numbers are obtained from the initial velocity data fitted to the Michaelis-Menten equation by NLR, and from Lineweaver-Burk (reciprocal; 1/v vs. 1/[mAb]) and Hanes-Woolf ([mAb]/v vs. [mAb]) linear transformations. K_s/V_{max} value, which denotes the rate at very early times, is the principal parameter for K_i calculation. K_s/V_{max} values were extracted from Michaelis-Menten equation as the K_s/V_{max} ratio. In Lineweaver-Burk reciprocal plot, K_s/V_{max} is defined by the gradient, and in Hanes-Woolf plot by the y-intercept. The kinetics of linear competitive inhibition is characterized by the increase of K_s/V_{max} and by unchanged V_{max}. This inhibition mode operates within a functional [I] range, and to delimit that range K_s/V_{max} values obtained by NLR, Lineweaver-Burk and Hanes-Woolf plots were computed and plotted against [I] series. Points which deviated from linearity were discarded for subsequent analyses, and only K_s/V_{max} values within the linear range were used to determine K_i from the linear fit of a K_s/V_{max} against [I] plot.

To compute the K_i constant, the time-course data of mAb-[I] binding were analyzed by seven different methods (for a more detailed explanation see [8*]). The simplest technique is to determine K_i by plotting K_s/V_{max} values obtained from Michaelis-Menten, Lineweaver-Burk and Hanes-Woolf methods, against the [I] series. K_i constant is derived from the linear fit of that plot.

The Dixon plot [37] graphs the reciprocal of the initial inhibition velocity (1/v) against the series of [I] at different [mAb]. K_i can be obtained by drawing a perpendicular from the median of the intersecting points to the x-axis, as described for the direct linear plot [38]. This method is quick and direct, but dispersion of the intersecting points of the line can limit its use.

Ascenzi's plot ($K_s/V_{max}/K_{s,0}/V_{max} = [I].K_i^{-1}$) is a useful to analyze competitive inhibition data computed as the mean K_s/V_{max} values obtained at different [I] divided by $K_{s,0}/V_{max}$ obtained without inhibitor. This normalized value plotted against [I], yields $1/K_i$ as slope. This method is convenient for comparing data obtained under different conditions [39].

$IC_{50}/i_{0.5}$, a parameter widely used by pharmacologists to quantitate the [I] that causes 50% inhibition, is a relative value that depends on assay conditions and mode of inhibition [40]. IC_{50} is measured by plotting the fractional activity of inhibition v_i/v_o (fraction of receptor-bound mAb at a given inhibitor concentration) against [I]. Data were fitted to an IC_{50} background-corrected equation by NLR, and the IC_{50} values obtained at different [I] replotted as a function of [mAb]. K_i was obtained as the y-axis intersection.

The inhibitor concentration for half inhibition ($i_{0.5}$) was also determined by graphing the initial velocity of mAb binding as [mAb]/v against [I]. This plot assigns the inhibition type, and generates a series of parallel lines that intercept the x-axis at points which give $i_{0.5}$ as a function of [mAb]. In the cases of competitive and mixed-competitive inhibition a secondary plot of $1/i_{0.5}$ vs. v_o/V_{max} (relative rate of the uninhibited reaction) yields a set of values whose fitted line intersects the y-axis at $1/K_i$. In competitive inhibition this line intercepts the x-axis at 1, and in mixed inhibition at x >1. In some plots the abscissa interception can be at x >1, suggesting some type of mixed inhibition effect. This is not unexpected as antibodies are bivalent, and mAb competing binding to inhibitor and receptor should differ from a single paratope-epitope interaction. A useful feature of Cortés method is that permits interconvert $IC_{50}/i_{0.5}$ relative values to inhibition constants [41].

The accuracy of antibody constant values depends to some extent on the methods used for data analysis [42]. Similarly to

how mAb k_{+1} was determined, we consider more appropriate to express K_i as an average number (mean ± 95% confidence limits) generated from individual K_i values obtained by the six evaluation techniques.

CONCLUSIONS

Knowledge of antibody kinetic (k_{+1}, k_{-1}) and equilibrium (K_i) constants allows prediction of antibody biological activity. To measure these parameters we described several ELISA kinetic methods based on the initial rate of mAb binding to its ligand. Data analysis were done by combining curvilinear regression procedures with standard kinetic plots. Although visual plots are outmoded given current computing capabilities, we believe that these techniques provide the researcher with a choice of analytical tools to highlight different approaches for analyzing molecular recognition. Graphic methods can appear cumbersome, but with the aid of modern computer packages, good quality plot are readily made. They are accessible, inexpensive, and allow visualization of experimental irregularities and thus, appropriate correction, as 'there is no single statistical tool that is as powerful as a well-chosen graph' [43].

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