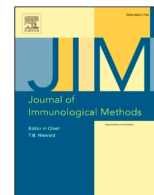
Journal of Immunological Methods 534 (2024) 113756

Contents lists available at ScienceDirect

Journal of Immunological Methods

journal homepage: www.elsevier.com/locate/jim



Description of a non-competitive ELISA based on time course analysis of ligand binding at saturation, and a direct method for calculating the affinity of monoclonal antibodies

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ARTICLE INFO

Keywords:
Monoclonal antibody affinity determination
Solid-phase time-course saturation ELISA
Noncompetitive inhibition kinetics

ABSTRACT

We present a time-course saturation ELISA for measuring the equilibrium constant of the monoclonal antibody (mAb) SIM 28 against horse radish peroxidase (HRP). The curves of HRP binding to a series of fixed mAb dilutions were plotted to completion, and the K_t (= K_s) value (time to occupy 50 % of the mAb paratopes) was determined for each mAb dilution and HRP concentration. Analysis of the kinetic mechanism of the reaction by Lineweaver-Burk and Hanes plots showed that the slope and y-intercept were affected, indicating that mAb ligand saturation follows non-competitive inhibition kinetics in this assay format. In this kinetics, the inhibition constant K_i (= K_d) is the time required to double the slope or halve the V_{max} of the Lineweaver-Burk plot. The K_t values of the time courses were doubled (2 x K_t) and normalized by dividing by the total reaction time to obtain a unitless factor which, when multiplied by the concentration of HRP, gives the K_i. The affinity constant of mAb SIM 28 was determined from ELISA data (n = 16) by three methods: i) doubling of K_t, ii) Beatty equation (K_{aff} = (n-1)/2 (n $[HRP']_t$ - $[HRP]_t$), and iii) SPR (Biacore) analysis. The calculated affinities (mean ± 95 % confidence limits) were i) $4.6 \pm 0.67 \times 10^{-9}$ M, ii) $K_{aff} = 0.23 \pm 0.03 \times 10^{9}$ M^{-1} ($K_d = 4.8 \pm 0.81 \times 10^{-9}$ M), and iii) $4.3 \pm 0.57 \times 10^{-9}$ M, respectively. The similar results obtained with the three different techniques indicate that this time-course saturation ELISA, combined with the double K_t method, is a repeatable and direct approach to mAb affinity determination.

1. Introduction

Immunoglobulins (antibodies) are blood serum glycoproteins that play an important protective role in the immunity of jawed vertebrates by neutralizing harmful substances and infectious agents. Antibodies contribute to host defense in several ways (Zinkernagel et al., 2001; Lenette et al., 2018), and the initial stage of their interaction with a ligand is the binding of their paratopes to the antigenic epitopes of the target molecule. The course of antibody interaction with a monovalent ligand follows the Langmuir adsorption isotherm, $\alpha = (K.C)/(1 + K.C)$, and the extent of the reaction "depends on the product of a concentration (C) and an association constant (K)" (Karush, 1970). The antibody concentration determines the rate of the reaction, and the affinity determines the sensitivity of the detection (Bachmann et al., 1997). The antibody affinity (K_d) and rate (k_{+1}) constants are two key parameters of antibody function (Mason and Williams, 1986) and important correlates of host protection against infection (Roost et al., 1995; Sattentau and Moore, 1995; Sterbeck et al., 2005; Bates et al., 2013; Dobaño et al., 2019).

Methods for the determination of antibody affinity can be categorized as homogeneous and heterogeneous (Steward and Chargelegue, 1996). In homogeneous methods, antibody-ligand interactions take place in solution and the reaction kinetics are described by the law of mass action. In heterogeneous techniques, one of the reactants is immobilized on a solid phase, and mass transport diffusion and steric considerations can limit antibody-ligand interactions (Goldberg and

Abbreviations: HRP, horseradish peroxidase; [HRP], HRP concentration; K_{aff}, apparent association constant with units (L/mol); K_d, apparent dissociation constant with units (mol/L), where $K_d = 1/K_{aff}$; K_i, inhibition (dissociation) constant in antibody-inhibitor interactions. The meaning and dimensions of K_i and K_d are identical; k_{+1}, second-order kinetic rate constant in units of M^{-1} s^{-1}; K_s, Michaelis rapid equilibrium constant; K_t, Michaelis constant in units of time; mAb, monoclonal antibody; NLR, nonlinear regression; SPR, surface plasmon resonance; $t_{1/2}$, time taken for the concentration of a reactant to fall to half its initial value.

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https://doi.org/10.1016/j.jim.2024.113756
Received 20 May 2024; Received in revised form 6 September 2024; Accepted 9 September 2024
Available online 13 September 2024
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Djavadi-Ohaniance, 1993; Nygren and Stenberg, 1989; Butler, 2000). For these reasons, heterogeneous methods such as solid phase ELISA are considered somewhat less reliable in estimating true antibody affinity constants (Pesce and Michael, 1992; Underwood, 1993).

Classical strategies for measuring antibody affinity are based on incubating known concentrations of antibody and ligand to equilibrium. From the amount of antibody or ligand bound or free, and knowledge of the initial concentrations of the reactants, the binding data are plotted as linear transformations of the mass law, and the equilibrium parameters are determined from the slope and y-intercept (Fazekas de St. Groth, 1979; H. M. V. Van Regenmortel, 1996). Methods that do not rely on data linearization have also been used to determine the affinity of monoclonal antibodies (Beatty et al., 1987a, 1987b; Raghava and Agrewala 1994; Loomans et al., 1995; Hajighasemi et al., 2004) and the distribution of affinity in antibody subpopulations (Nieto et al., 1984; Rath et al., 1988). Currently, biosensor technology allows real-time data acquisition and rapid return of antibody kinetic and equilibrium data, but this technique requires expensive instrumentation. In contrast, ELISA methods are inexpensive, simple, and can be developed in a variety of formats. As a result, solid-phase ELISA has been widely used to determine the apparent affinity constant of mAbs (Hoylaerts et al., 1990; Seligman, 1994; Fuchs et al., 1995; Orosz and Ovádi, 2002; Bobrovnik et al., 2005; Toraño et al., 2021).

To improve existing methods for assessing antibody affinity, we describe a heterogeneous non-competitive saturation ELISA based on time-course analysis of antigen binding to a series of plate-bound mAb dilutions. In addition, we provide a simple approach for calculating the mAb affinity constant. This method is based on the finding that the interaction of a bivalent mAb with HRP (mAb-HRP_2) exhibits the kinetics of a non-competitive inhibition reaction. This mechanism provides a direct way to determine the mAb inhibition constant (K_i) as the amount of inhibitor required to double the slope (K_t) or halve the Vmax of the Lineweaver-Burk plot.

To assess the reliability of this method, we compared the affinity of Ab SIM 28 determined by three different techniques. The similar affinity results obtained indicate that the time-course saturation ELISA and the double K_t method provide a reliable and straightforward method for determining mAb affinity.

2. Materials and methods

2.1. Reagents and buffers

ELISA 96-well microplates (MaxiSorp 442,404; Nunc, Roskilde, DK) were used. Reagents included Tween-20, ortho-phenylenediamine (OPD), bovine serum albumin (BSA), and horseradish peroxidase (HRP; P-6782, type VI-A), all from Sigma-Aldrich (St. Louis MO, USA). Other reagents were HRP substrate (0.4 mg/ml OPD in 50 mM phosphate-citrate buffer pH 4.8, with 33 % H_2O_2 (diluted 1/1000), phosphate-buffered saline (PBS; 10 mM phosphate, 0.15 M NaCl, pH 7.4), PBST (PBS pH 7.4, with 0.05 % Tween 20), and BSA blocking buffer (2 % BSA in PBST).

2.2. Antibodies and antigens

Sheep anti-mouse IgG (ShaM) was isolated from hyperimmune serum and processed as described (Domínguez et al., 2019). mAb SIM 28–31.8.21 anti-HRP (IgG_1) was prepared in-house as described (Monedero et al., 2019), grown in culture supernatant (~22 μg/ml) supplemented with 0.05 % NaN_3 and stored at 4 °C. Horseradish peroxidase (HRP; P-6782, type VI-A) was purchased from Sigma-Aldrich (St. Louis MO, USA).

2.3. Methods of analysis

The mAb-ligand interaction is assumed to follow Michaelis-Menten rapid equilibrium kinetics. To ensure linearity of HRP binding with time, data were obtained from initial velocity measurements ($A_{492\ nm}$, 1 min). Ligand concentrations and mAb dilutions were prepared from master stock solutions as described (Bisswanger, 2011). Setting the range of ligand/mAb ratios for the time courses requires knowledge of the ligand concentration ([HRP]) and an estimate of the amount of [mAb] in the culture supernatant. To generate the time course curves, an excess of sheep anti-globulin (ShaM) was used to bind a series of mAb doubling dilutions at ratios of 1:-2:- 4:- 8 to the plate. The mAb dilutions were adjusted so that the maximum ligand binding ($A_{492\ nm}$) was approximately 1.2–1.4 units at the lower mAb dilution. To generate the hyperbolic time courses, ligand concentrations above those of mAb ([HRP] > [mAb]) were used. Two ligand concentrations were used, one in relative excess (9.2 nM) and the other in substantial excess (34 nM). The time course data allowed the estimation of the K_t values of the different dilution curves needed to calculate the mAb constant.

HRP binding rate is reported as the average of duplicate or triplicate observations from reactions developed at room temperature (~22 °C). Four 1:150 (Ab_{150}), 1:300 (Ab_{300}), 1:600 (Ab_{600}), and 1:1200 (Ab_{1200}) dilutions of mAb, each half the size of the other, e.g., $Ab_{150} = 2\ Ab_{300} = 4\ Ab_{600} = 8\ Ab_{1200}$, were typically added to the plate wells of these ELISAs and reacted with two doses of HRP: 9.2 nM (400 ng/ml) and 34 nM (1500 ng/ml). The time at which the curve reached its plateau (equilibrium) was estimated by measuring the amount of HRP bound after doubling the incubation time; if the increase in HRP binding was <10 %, the reaction was considered complete (Mason and Williams, 1986). The proportion (%) of mAb bound to HRP during the incubation times (T_i) was determined as described by plotting $1/1+ K_t:T_i$ versus total reaction time (T).

The HRP binding data were fitted by a non-linear Michaelis-Menten equation to obtain the K_t values of the time courses. It is simpler and more accurate to use the Michaelis-Menten equation to calculate the K_t values than to determine the bound ligand from the OD-50 of a sigmoid curve. Alternatively, K_t values can be obtained with identical results by fitting the HRP binding data to a single saturation curve equation where the amount of HRP bound is plotted as a function of time. Conversion of the K_t values to dimensionless numbers by dividing by the total reaction time (T) resulted in a set of normalized parameters F (= K_t/T) which, when multiplied by the [HRP] of each time course, yielded the amount of HRP bound to the antibody. In addition, mAb affinity was calculated using Beatty's equation, $K_{aff} = (n-1)/2\ (n\ [HRP']_t - [HRP]_t)$ (Beatty et al., 1987a, 1987b), where $[HRP']_t$ and $[HRP]_t$ are the amount of HRP bound at K_t of time courses taken in wells coated with two mAb dilutions, and [HRP']t is the HRP bound at the higher of the two dilutions.

To characterize the kinetics of mAb SIM 28-HRP interaction, HRP binding data were linearized in reciprocal (1/HRP binding rate versus 1/t (time)) Lineweaver-Burk, and (t/HRP binding rate versus t) Hanes plots. To reduce error amplification during linearization, V_{max} and K_s values derived from nonlinear regression of untransformed data fitted to the Michaelis-Menten equation were inserted into the double reciprocal and Hanes equations and plotted as a linear regression fit (Copeland, 2000).

Analysis of these plots showed that their lines differed in slope and y-intercept; in addition, the lines of the plots intersected the -x-axis at a common point to the left of the ordinate (except in the case of experimental variation), indicating a non-competitive inhibition-type mechanism.

2.4. Time course of HRP binding to mAb SIM 28

Under equilibrium conditions, the mAb-HRP complex formed must remain constant over time. We timed the reaction to reach equilibrium by measuring the amount of HRP bound to mAb at various intervals over a ninety-minute period. The experiment was performed by incubating each of the four mAb dilutions (1:150, 1:300, 1:600, and 1:1200) with three [HRP]. Three ELISA plates were coated with 50 μl/well of ShaM (4

μg/ml in PBS; 15 min, 37 °C), washed three times with PBST, and blocked (15 min, 37 °C) with 100 μl/well of 2 % BSA/PBST. The plates were again washed three times with PBST and 50 μl/well (duplicates) of a dilution series of SIM 28 culture supernatant in PBST was added to ShaM coated wells in rows 1–2 (1:150 dilution), 3–4 (1:300 dilution), 5–6 (1:600 dilution), and 7–8 (1:1200 dilution) and incubated (1 h, 37 °C). After incubation, the plates were washed three times with PBST and 25 μl PBST was added to all wells. The three identical plates run in parallel differed only in the final [HRP] added: 2.3 nM (100 ng/ml), 9.2 nM (400 ng/ml), and 34 nM (1500 ng/ml). The reaction was initiated by adding 25 μl of twofold concentrated HRP to the wells in column 12 (time 0). At predetermined intervals, 25 μl of 2 x HRP was added to the wells in columns 11 (after 30 min), 10 (50 min), 9 (60 min), 8 (70 min), 7 (75 min), 6 (78 min), 5 (81 min), 4 (84 min), 3 (87 min), 2 (89 min), and 1 (90 min). The final incubation times were 90 min (column 12), 60 min (column 11), 40 min (column 10), 30 min (column 9), 20 min (column 8), 15 min (column 7), 12 min (column 6), 9 min (column 5), 6 min (column 4), 3 min (column 3), 1 min (column 2), and 0 min (column 1). Incubation was terminated by immersing the plates three times in PBST and draining by blotting on a paper towel between each immersion. The HRP reaction was developed (1 min) by adding 70 lL/well HRP substrate (1 mg/ml) and terminating with 30 μl/well 3 N H_2SO_4. The reaction product was measured at absorbance ($A_{492\ nm}$).

2.5. Surface plasmon resonance (SPR) analysis

The affinity constant (K_d) of the mAb SIM 28-HRP reaction was measured on a Biacore X100 instrument (GE Healthcare, Spain). Goat anti-mouse IgG antibodies were covalently bound to sample and reference cells of a CM5 chip at 10,000–12,000 response units (RU), according to the manufacturer's instructions. Approximately 150 RU of mAb SIM 28 were captured with goat anti-mouse IgG antibodies and eight different [HRP] ranging from 7.81 to 1000 nM were sequentially injected at a flow rate of 30 μl/min. Antibody kinetic parameters were calculated by fitting the binding data to a 1:1 L binding model.

Excel and GraFit software (v. 7) (Leatherbarrow, 2009) were used for computational analyses of NLR, linear fit data, and figure preparation.

3. Results

3.1. Determination of the response time required to reach the binding plateau

We incubated three doses of HRP (2.3 nM, 9.2 nM, and 34 nM) with four mAb dilutions across an eightfold concentration range (1:150, 1:300, 1:600, and 1:1200) for ninety minutes (Fig. 1).

At 2.3 nM, the molar ratio of HRP to mAb varies from 2.3:1 (at 1:150 dilution) to 18:1 (at 1:1200 dilution), and the average saturation values of the four mAb dilutions were 61 % and 66 % after 3600 and 5400 s of incubation, respectively (Fig. 1A). HRP binding increased by 14.4 % between 60 and 90 min, but the time courses did not plateau. In cases where the ligand concentration was in limited excess of the mAb, the time courses did not plateau and the K_t values were inaccurate.

This was not the case for HRP doses of 9.2 nM (Fig. 1B) and 34 nM (Fig. 1C). The mean increase between 60 and 90 min of incubation was 6 % (at 9.2 nM) and 3.9 % (at 34 nM), respectively. After 60 min, the time courses were considered complete with mean average antibody saturation of 83 % and 94 %, respectively.

Proper assay conditions require that [HRP] > [mAb]. To determine whether the concentration of the limiting reactant (mAb) might affect the outcome of the K_d (Jarmoskaite et al., 2020), we analyzed data from reactions performed in sufficient to large excess of HRP over [mAb]. Using HRP doses of 9.2 nM and 34 nM, respectively, the molar ratios of HRP to mAb varied from 9.2:1 (at 1:150 dilution) to 72:1 (at 1:1200 dilution) and from 34:1 (at 1:150 dilution) to 272:1 (at 1:1200 dilution). When [HRP] was sufficiently high relative to [mAb], the K_{aff} values obtained from these experiments showed that the limiting reactant had no effect on the value of the affinity constant.

3.2. Kinetics of mAb SIM 28-HRP interaction

To characterize the kinetic mechanism of this reaction, we analyzed the data from the time courses shown in Fig. 1B and C by plotting the HRP binding rate versus time using the reciprocal (Lineweaver-Burk) (Fig. 2A) and Hanes (Fig. 2B) plots.

In these plots, their lines differ in slope and y-intercept, which

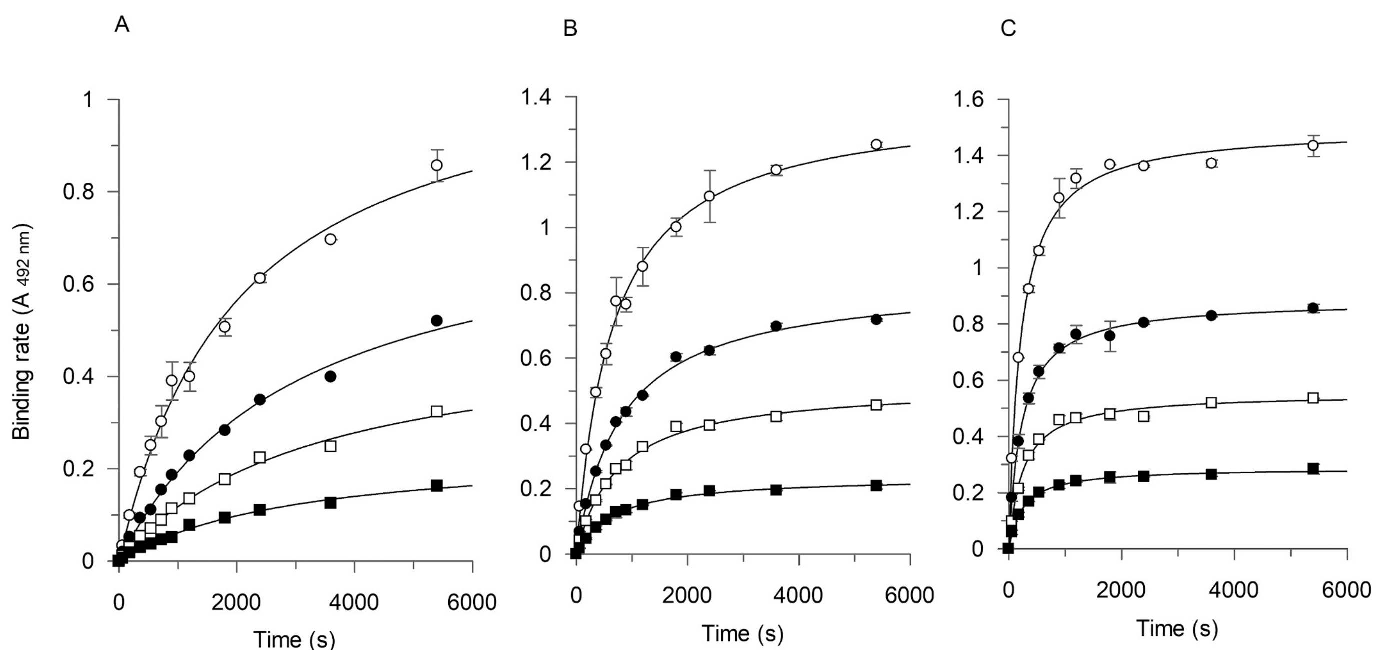

Fig. 1. Progress curves of HRP binding to mAb SIM 28 over a 90-min incubation period. Four dilutions of mAb SIM 28, 1:150 (○), 1:300 (●), 1:600 (□), and 1:1200 (■), were incubated for 90 min with three different dosages of HRP, 2.3 nM (panel A), 9.2 nM (panel B), and 34 nM (panel C). The progress curves show the HRP binding rate (mean ± standard error) during the 90-min incubation. Assay conditions as described in section 2.4.

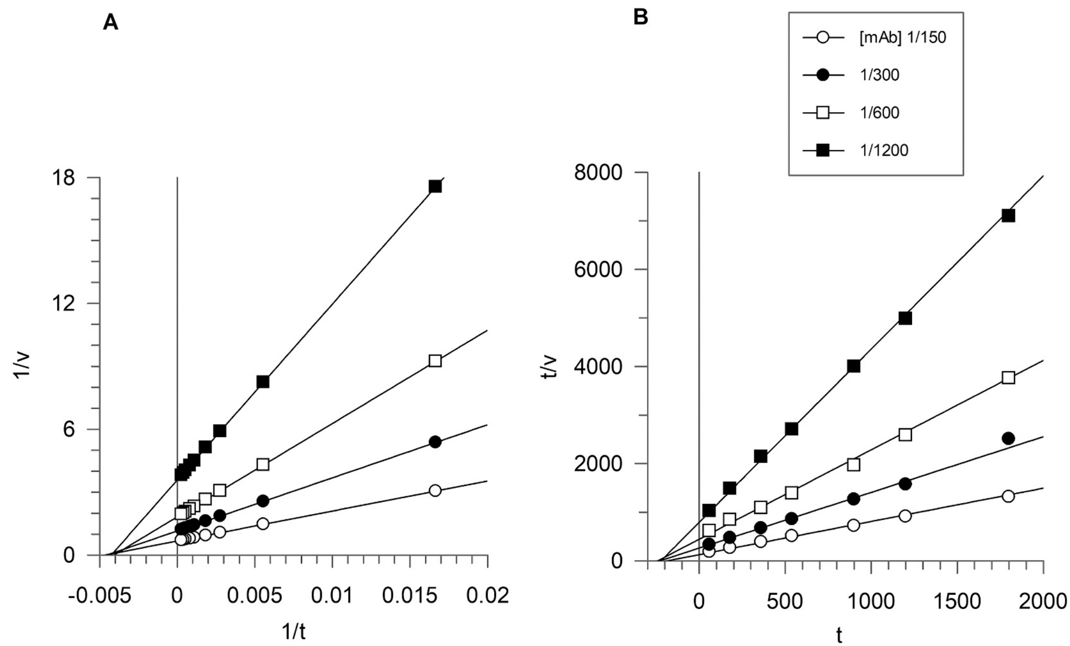

Fig. 2. Kinetic mechanism of the SIM 28-HRP reaction time course. HRP binding rate data at the different antibody dilutions shown in Table 1 taken from the progress curves in Fig. 1C, were linearized by Lineweaver-Burk (Fig. 2A) and Hanes (Fig. 2B) plots. The lines of the plots differ in slope and y-intercept, which increase proportionally with mAb dilution and intersect the -x-axis at a point to the left of the ordinate. This pattern is characteristic of linear non-competitive inhibition kinetics involving a binding agent (mAb) with two binding sites of equal affinity.

Table 1
Calculation of mAb SIM 28 affinity constants using (A) the double Kt method and (B) the Beatty equation. Data include HRP binding rate ($A_{492\ nm}$, 1 min), reaction parameters (V_{max}, Kt, total reaction time (T), and ligand concentration ([HRP])), and the steps required to calculate affinity values. Measurement of Ki by the double Kt method (A) requires only the division of the 2 x Kt value by T. The calculation of K_{aff} using the Beatty eq. (B) has two parts. First, the [HRP] bound is obtained as the product of the F factor and the [HRP] bound at each mAb dilution. Second, mAb K_{aff} is calculated by fitting the bound [HRP] to the Beatty equation using the mAb dilution ratios listed in the table.

	mAb dilution			
Time (s)	1/150 Rate A_{492nm}	1/300 Rate A_{492nm}	1/600 Rate A_{492nm}	1/1200 Rate A_{492nm}
0	0	0	0	0
60	0.3208	0.1813	0.0978	0.0585
180	0.6792	0.3820	0.2134	0.1212
360	0.9237	0.5352	0.3315	0.1681
540	1.0594	0.6294	0.3884	0.1998
900	1.2475	0.7129	0.4577	0.2250
1200	1.3172	0.7624	0.4645	0.2407
1800	1.3668	0.7168	0.4788	0.2537
2460	1.3612	0.8038	0.4693	0.2559
3600	1.3709	0.8281	0.5175	0.2629

A) Calculation of the affinity by the double Kt method				
V_{max}	1.5	0.87	0.55	0.28
K_t	215	220	245	235
T (total time)	3600	3600	3600	3600
2 x K_t	430	440	490	470
2 x K_t /T	0.12	0.12	0.14	0.13
[HRP] nM	34	34	34	34
HRP bound	4.1	4.1	4.8	4.4
Ki = 4.3 ± 0.53 [1]				

B) Calculation of the affinity with the Beatty equation				
V_{max}	1.5	0.87	0.55	0.28
K_t	215	220	245	235
T (total time)	3600	3600	3600	3600
F = K_t/T	0.06	0.061	0.068	0.065
[HRP] nM	34	34	34	34
HRP bound (F x [HRP])	2.0	2.1	2.3	2.2
K_{aff} = n-1/2 (n [HRP']t -[HRP]t])				
mAb (dilution ratio)	K_{aff} x10^9 M^{-1}		K_d x 10^{-9} M	
150:300, *n* = 2	0.23		4.4	
150:600, n = 4	0.20		4.8	
150:1200, *n* = 8	0.22		4.5	
300:600, n = 2	0.20		5.0	
300:1200, *n* = 4	0.22		4.5	
600:1200, n = 2	0.24		4.2	
Affinity	0.22 ± 0.021 [1]		4.6 ± 0.37 [1]	

[1] Mean ± 95 % CL

HRP binding data.

increase proportionally to the mAb dilution. In both cases, V_{max} is reduced by a factor close to 2, which causes the slopes and y-intercepts to increase by the same factor. The change in V_{max} has no effect on K_s, which remains unchanged, and the lines of the plots intersect the -x-axis at a point to the left of the ordinate. This pattern is characteristic of linear noncompetitive inhibition kinetics involving a compound with two binding sites of equal affinity. In this kinetics, K_s remains constant as V_{max} decreases by a factor of $(1 + [I]/K_i)$. When [I] is equal to K_i, $(1 + [I]/K_i)$ is equal to 2, which halves V_{max} and doubles the slope (K_s/V_{max}) of the reciprocal plot (Haldane, 1930: Cleland, 1963). The inhibition constant is derived from the time required to double the slope or halve the V_{max} of the Lineweaver-Burk plot. To calculate K_i, the K_t value of each time course was doubled (2 x K_t) and divided by the total reaction time to generate a unitless factor that was multiplied by the concentration of HRP to give K_i. We refer to this procedure as the double K_t method.

3.3. Determination of mAb SIM 28 affinity for HRP

To calculate the affinity of mAb SIM 28 for HRP, we use data from an ELISA performed for 3600 s incubation with two HRP doses of 9.2 (Fig. 1B) and 34 nM (Fig. 1C) (section 2.4). For simplicity, only data obtained with 34 nM HRP will be discussed.

Determination of antibody affinity using the double K_t method is straightforward, requiring only that the K_t value be doubled and divided by the total reaction time. The product of K_t/T x HRP dose used in the time course gives the amount of HRP bound at that mAb dilution, which is the K_i. The apparent affinity constant of SIM 28 is the average K_i of the four antibody dilutions (Table 1 A).

To calculate the mAb K_{aff} using the Beatty equation, the K_t values of the time courses were first converted to dimensionless factors (K_t/T), which, when multiplied by the HRP dose, yielded the amount of HRP bound at each mAb dilution (Table 1B). The four mAb dilutions were combined to generate the “n” values for the equation: $Ab_{150}/Ab_{300} = 2$, $Ab_{150}/Ab_{600} = 4$, $Ab_{150}/Ab_{1200} = 8$, $Ab_{300}/Ab_{600} = 2$, $Ab_{300}/Ab_{1200} = 4$, and $Ab_{600}/Ab_{1200} = 2$. The HRP bound data were then calculated using the equation $[K_{aff} = (n-1)/2 (n [HRP']_t - [HRP]_t)]$. The final average affinity result was obtained from six K_t values from each HRP concentration and a total of twelve recordings (Table 1B).

The antibody affinity constant can also be inferred from the y-intercept and slope of the Lineaweaver-Burk (Fig. 2A) and Hanes (Fig. 2B) plots. K_i values were determined as the mean of the slope intersections on the -t axis as $-1/K_t$ (Fig. 2A) or on the -t axis as - K_t (Fig. 2B). In addition, K_i can also be obtained from the y-intercept values of these plots. Using V_{max} values to calculate K_i gives the same average result as the double K_t method, but the variance is too large because V_{max} decreases proportionally with mAb dilution. Table 2 summarizes the calculations performed to determine mAb K_i using data linearized by Lineweaver-Burk (Table 2A) and Hanes (Table 2B) plots.

3.4. Summary of mAb SIM 28 affinity values

The time course saturation data to determine the affinity of mAb SIM 28 were analyzed by two methods: SPR technology (Biacore) and ELISA combined with the 2 x K_t method and the Beatty equation. The equilibrium and kinetic constants of mAb SIM 28 obtained by Biacore were (mean ± 95 % CL, $n = 4$) $K_d = 4.34 \pm 0.57 \times 10^{-9}$ M (K_{aff} (= $1/K_d$) = $0.23 \pm 0.064 \times 10^{9}$ M^{-1}), $k_{+1} = 0.87 \pm 0.72 \times 10^{5}$ M^{-1} s^{-1} and $k_{-1} = 3.8 \pm 0.06 \times 10^{-4}$ s^{-1}. (Table 3) In turn, the ELISA data were collected over a period of three months from eight parallel ELISA experiments (n = 16) performed with two HRP doses in low (9.2 nM) and high excess (34 nM) and an incubation time of 3600 s yielding Ki = 4.6 ± 0.67 × 10–9 M. In ELISA, the concentration of ligand used has a small effect on the time course K_t values, which is reflected in the apparent K_i. Nevertheless, the similar results and the range of variation of the values obtained by SPR and ELISA techniques support the accuracy of the saturation time course and double K_t methods.

Table 2

Determination of mAb SIM 28 K_i using HRP binding data linearly transformed with Lineweaver-Burke (A) and Hanes (B) plots. The HRP binding data shown in Table 1, derived from the progress curves in Fig. 1C, were linearized as 1/v vs. 1/t (A) and t/v vs. t (B). The intercept/slope ratios of these plots give the values of $-1/K_t$ in Lineweaver-Burke (A) and -K_t in Hanes (B) plots at the intersection of the -x axis. The amount of HRP bound is obtained as in the 2 x K_t method by the product of [HRP] x (2 x K_t/T). The SIM 28 apparent K_i is calculated as the mean of the four K_i results for each assay.

A) Lineweaver-Burk linearized rate data.						B) Hanes linearized rate data.				
		1/v				t/v				
time (s)	1/t	1/150 Rate A_{492nm}	1/300 Rate A_{492nm}	1/600 Rate A_{492nm}	1/1200 Rate A_{492nm}	time (s)	1/150 Rate A_{492nm}	1/300 Rate A_{492nm}	1/600 Rate A_{492nm}	1/1200 Rate A_{492nm}
0	0	0	0	0	0	0	0	0	0	0
60	0.0167	3.12	5.52	10.22	17.1	60	187	331	613	1026
180	0.0056	1.47	2.62	4.69	8.25	180	265	471	843	1485
360	0.0028	1.08	1.87	3.02	5.95	360	390	673	1086	2141
540	0.0019	0.94	1.59	2.57	5.0	540	510	858	1390	2703
900	0.0011	0.80	1.40	2.18	4.44	900	721	1262	1967	4000
1200	0.0008	0.76	1.31	2.15	4.15	1200	911	1574	2584	4985
1800	0.0006	0.73	1.39	2.09	3.94	1800	1317	2511	3759	7095
2460	0.0004	0.73	1.24	2.13	3.91	2460	1807	3060	5242	9611
3600	0.0003	0.73	1.21	1.93	3.80	3600	2626	4347	6956	13,693
Intercept		0.57	0.98	1.5	3.07	Intercept	126	262	443	792
Slope		156	277	531	861	Slope	0.685	1.15	1.84	3.57
Intercept/slope = K_t		0.0037	0.0035	0.0028	0.0036	Interc/slope = K_t	184	228	241	222
K_t		270	286	354	278	2 xK_t	368	456	482	444
2 x K_t		540	572	708	556	T (total time)	3600	3600	3600	3600
T (total time)		3600	3600	3600	3600	2 x K_t/T	0.102	0.127	0.134	0.123
2 x K_t/T		0.15	0.16	0.20	0.15	HRP nM	34	34	34	34
HRP nM		34	34	34	34	HRP bound (K_i)	3.47	4.32	4.56	4.18
HRP bound		5.1	5.4	6.8	5.1					
K_i = 5.6 ± 1.2 nM [1]						**K_i = 4.1 ± 0.73 nM** [1]				

[1] Mean ± 95 % CL

Table 3
Apparent affinity constants (K_{aff}, K_i and K_d) (mean ± 95 % confidence limits, an range) of mAb SIM 28 obtained by SPR and from each of two ELISA series (n = 8) performed in parallel using two doses of HRP 9.2 nM and 34 nM and an incubation time of 3600 s. Affinity K_{aff} values ($K_d = 1/_{Kaff}$) were calculated using the Beatty equation, and K_i and K_d were determined using the 2 x K_t method and Biacore analysis, respectively. Final apparent affinity constants were calculated as the average of the total number of assays performed (n = 16).

Number of assays	[HRP] nM	K_{aff} nM^{-1} (range)	K_d nM (range)	K_i nM (range)
ELISA (n = 8)	9,2	0.20 ± 0.05 [1]	5.6 ± 2.1 [1]	5.5 ± 1.5 [1]
ELISA (n = 8)	34	0.26 ± 0.05 [1]	4.1 ± 0.80 [1]	3.8 ± 0.73 [1]
ELISA (n = 16)	9.2 + 34	0.23 ± 0.03 [1] (0.20–0.26) [2]	4.8 ± 0.81 [1] (4–5.6) [2]	4.6 ± 0.67 [1] (3.9–5.3) [2]
Biacore (n = 4)	7.8–1000	0.23 ± 0.064 [1] (0.17–0.29) [2]	4.3 ± 0.57 [1] (3.7–4.9) [2]	

[1] mean ± % 95 % CL.
[2] range.

4. Discussion

The antibody affinity constant K_d (= k_{-1}/k_{+1}) can be determined by measuring the concentrations of the reactants at equilibrium or by kinetic studies to quantify the rate constants at which the reactants bind and dissociate. Using kinetic methods, only SPR-based techniques currently allow the determination of antibody kinetic and equilibrium data from a single experiment. In this report, we describe a time-course ELISA that integrates the equilibrium and kinetic aspects of both approaches with a method that simplifies the measurement of the affinity constant of the antibody.

Homogeneous competitive immunoassays are considered to be the most accurate ELISA approaches for measuring mAb affinity by conventional methods. Friguet (Friguet et al., 1985) developed such an indirect ELISA method, which has been widely adopted by other researchers (Robbins et al., 1988; Bator and Reading, 1989; Seligman, 1994; Bobrovnik et al., 2010), although some shortcomings have been described (Stevens, 1987; Bobrovnik et al., 2005).

Affinity data derived from heterogeneous ELISA are not considered true affinity measurements because the immobilization of a reactant on the solid phase does not conform to thermodynamic theory; however, heterogeneous ELISA techniques are inexpensive, easy to use, and available in a variety of formats; for these reasons, they have been widely used for antibody affinity determination with reasonable results.

Here we describe a solid-phase ELISA to determine the affinity constant of mAb SIM 28 for HRP, for which we have adapted some methodological features of a non-competitive assay (Beatty et al., 1987a, 1987b). These authors quantified the amount of mAb binding at equilibrium from the OD-50 values of curves generated by plotting the antibody bound at different antigen concentrations against the $\log_{10}$ of the antibody dilution. To calculate the mAb affinity constant, they developed the equation $K_{aff} = (n-1)/2\ (n\ [Ab']_t - [Ab]_t)$, where $[Ab']_t$ and $[Ab]_t$ are the antibody bound at the OD-50 point of the sigmoid curves generated in wells coated with two amounts of antigen, where $[Ab]_t = 2\ [Ab']_t$.

The time-course ELISA we developed can be compared to the progress curve of an enzyme-catalyzed reaction in which the substrate concentration is replaced by time. Section 2.3 describes the reaction conditions (temperature, incubation time) and reactant concentrations (mAb and HRP) that determine the reaction rate and the limiting binding, respectively. The fraction of mAb paratopes occupied by HRP is given by the Michaelis constant of the progress curve expressed in units of time (K_t), and the time to reach saturation (60 min) was determined after confirming that the amount of HRP bound did not change significantly (<10 %) by nearly doubling the incubation time.

Adsorption is the most common ELISA technique used to bind antigens to the plate. This practice can significantly alter the biological activity of the reactant (Butler, 2000), and in competitive ELISA, the type and solid phase density of the coated antigen can significantly affect the antibody K_d value (Hetherington, 1990; Seligman, 1994). In the Beatty method, antigen coating is a key procedure in which plates are coated with a series of twofold dilutions of antigen concentrations. Using this method, it is nearly impossible to determine the amount of functional antigen adsorbed on the wells (Loomans et al., 1995). Because of the symmetry of the SIM 28-HRP interaction, we chose to measure antigen binding to the mAb captured on the plate by covering the wells with anti-globulin (ShaM) in excess of the mAb. ShaM prevents denaturation of the mAb by adsorption and, when present in excess, quantitatively binds the mAb, allowing accurate determination of the bound mAb. This is important because in order to calculate the [mAb]/[HRP] ratio for the time courses, it is necessary to know the approximate amount of mAb bound to the wells.

In these experiments we have used ShaM to capture the mAb. However, any antiglobulin from another species can be used provided that cross-reactivity to a potential antibody reporter (either biotinylated or enzyme conjugated) has been removed. Protein A and G may be used to attach the mAb to the plate if the mAb subclass binds to these reagents. If Protein A or G is used, the reporter molecule cannot be an antibody.

During hybridoma biosynthesis and subsequent handling, a fraction of the antibody sample becomes functionally inactive. Therefore, when using antibody as a measuring component, it is important to know its immunoreactive fraction because inactive molecules will alter the K_d value (Azimzadeh and Van Regenmortel, 1991). Inactive antibodies affect the Friguet and Beatty assays, but do not affect this time course format. We choose ligands as the titrating component because antibodies can lose their specificity even if they retain most of their antigenic characteristics, while ligands are less likely to become inactive unless there is a change that affects the binding epitope.

Analysis of the HRP binding data showed that the V_{max} of the time courses decreased with mAb dilution while the K_s values remained unchanged. In addition, linearization of the binding data with Lineweaver-Burk and Hanes plots showed that their slopes and y-intercepts increased by the same factor and that their lines intersected the -x-axis at a point to the left of the ordinate. This kinetic behavior is representative of a non-competitive inhibition reaction between a ligand and an antibody with two paratopes P1 and P2 of identical affinity ($K_i^{P1} = K_i\ ^{P2}$). To analogize mAb-ligand saturation to a non-competitive inhibition reaction, we considered the time against which the binding rate is titrated as the surrogate substrate and the mAb dilutions as the inhibitor concentrations, keeping in mind that at high mAb dilutions there is less binding, which can be equated to a higher inhibitor concentration. "The net effect of a non-competitive inhibitor is to make it appear as if less total enzyme is present" (Segel, 1975). We believe this is what is happening in this assay. Increasing the dilution of the antibody (surrogate inhibitor) decreases the amount of mAb (binding enzyme) present, simulating the effect of a non-competitive inhibition mechanism.

The equation for this reaction is, $v = V_{max}.\ S\ /K_s\ (1 + [I]/K_i\ P^1) + [S]\ (1 + [I]/K_i\ P^2)$. Under fast equilibrium conditions, when $K_i^{P1} = K_i\ ^{P2}$, it can be written as $v/V_{max} = [S]\ /\ ([S] + K_s)\ (1 + [I]/K_i)$. Division of both sides of the equation by $(1 + [I]/K_i)$ gives $v/V_{max}/(1 + [I]/K_i) = [S]/K_s + [S]$. In this expression, the apparent V_{max} (V_{max}^{app}) becomes $V_{max}/(1 + [I]/K_i)$, the value of which decreases by a factor of $(1 + [I]/K_i)$ as the inhibitor concentration increases. When the [I] concentration is equal to K_i, the apparent V_{max} is halved, which doubles the slope ($K_s\ /V_{max}$) of the reciprocal plot.

This kinetic mechanism provides a rapid method for determining mAb inhibition constants by doubling the K_t value of each time course (2 x K_t) and dividing by the total reaction time. This produces a unitless factor which, when multiplied by the concentration of HRP, gives the K_i of each time course dilution. The average K_i of the four time courses is used as the apparent mAb Ki.

Different reaction formats may be subject to different kinetic

mechanisms. For example, competition between mAb and free ligand for binding to solid-phase bound antigen exhibits competitive inhibition kinetics (Toraño et al., 2021), whereas the saturation of solid-phase bound mAb by free ligand shown in this study follows a non-competitive inhibition mechanism. Without the use of enzyme kinetic methods, we would not be able to identify the mechanism of mAb-ligand interaction in this type of assay, as competitive and non-competitive mechanisms are indistinguishable by graphical analysis of binding data (Bisswanger, 2017).

The affinity data obtained by ELISA and SPR techniques show reasonable similarity confirming the accuracy of measurements by both procedures. To evaluate the functional relevance of the small differences in SIM 28 affinity obtained by both methods, we compared the half-lives ($t_{1/2}$) of the SIM 28-HRP complexes based on their K_d constants. Using the dissociation rate constant ($k_{-1} = 3.8 \times 10^{-4}$ s^{-1}) and the K_d values from the Biacore analysis, the $t_{1/2}$ of the SIM 28-HRP complex was calculated to be 30.4 min. The $t_{½}$ values derived from the double K_t method and Beatty's equation were 29.2 min and 30.4 min, respectively, which are 98 % identical to the time estimated from Biacore. Even a one-unit discrepancy in K_d values would represent a loss of only 6.1 min (20.2 %) in the life of the complex, which would likely have a negligible effect on antibody performance.

A good analytical method should be easy to use and quick to perform. This time-course ELISA is a basic procedure that can be developed in approximately 3.5 h. The enzymatic activity of the ligand (HRP) in this assay allows the experiment to be completed more quickly. However, any conventional ligand can be assayed by this method using ligand biotinylation, a simple and gentle method for labeling most antigens (Moreno et al., 2022). In this case, the assay wouldn't take much longer than four hours.

5. Conclusions

Antibody affinity is an important factor in the evaluation of immunoglobulin function. To improve current methods for assessing affinity constants, we have developed a non-competitive time-course ELISA that is run to completion with an excess of ligand over mAb. This reaction format follows non-competitive inhibition kinetics and provides a rapid approach, the double K_t method, for determining mAb inhibition constants. The time-course saturation ELISA is easy to perform, inexpensive, and has fewer requirements than equivalent techniques. In addition, the similar results obtained from SPR analysis and this method confirm its accuracy for antibody affinity determination.

CRediT authorship contribution statement

Alfredo Toraño: Writing – review & editing, Writing – original draft, Methodology, Investigation, Conceptualization. **Inmaculada Moreno:** Writing – review & editing, Methodology, Investigation. **José Antonio Infantes:** Investigation. **Mercedes Domínguez:** Methodology, Investigation, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare no conflict of interest.

Acknowledgements

The authors thank Nela Díez, Ana Belén Martin, and Soledad Crespo for the development of the monoclonal antibodies and culture supernatants.

This work was partially supported by grants from the Ministry of Economy, Industry and Competitiveness of Spain (FIS PI13/011446 and RTC-2016-4635-1), which had no role in the study design.

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