Analytical Biochemistry 429 (2012) 58–69

Contents lists available at SciVerse ScienceDirect

Analytical Biochemistry

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

Biacore surface matrix effects on the binding kinetics and affinity of an antigen/antibody complex

Andrew W. Drake [1], Margaret L. Tang, Giuseppe A. Papalia [2], Gregory Landes [3], Mary Haak-Frendscho [1], Scott L. Klakamp [*,4]

Takeda San Francisco, 285 E. Grand Ave. South San Francisco, CA 94080, USA

ARTICLE INFO

Article history:
Received 25 April 2012
Received in revised form 20 June 2012
Accepted 25 June 2012
Available online 2 July 2012

Keywords:
Biacore
Surface plasmon resonance
Kinetic exclusion assay (KinExA)
SPR
Antigen–antibody complexes
Negatively charged dextran layer
Kinetic rate constants
Affinity
Equilibrium dissociation constants

ABSTRACT

To characterize a proprietary therapeutic monoclonal antibody (mAb) candidate, a rigorous biophysical study consisting of 53 Biacore and kinetic exclusion assay (KinExA) experiments was undertaken on the therapeutic mAb complexing with its target antigen. Unexpectedly, the observed binding kinetics depended on the chip used, suggesting that the negatively charged carboxyl groups on CM5, CM4, and C1 chips were adversely affecting the Biacore kinetic results. To study this hypothesis, Biacore solution-phase and KinExA equilibrium titrations, as well as KinExA kinetic measurements, were performed to establish accurate values for the affinity and kinetic rate constants of the binding reaction between antigen and mAb. The results revealed that as the negative charge on the biosensor surface decreased, the binding kinetics and K_D approached the accurate binding parameters more closely when measured in solution. Two potential causes for the artifactual Biacore surface-based measurements are (i) steric hindrance of antigen binding arising from an interaction of the negatively charged carboxymethyldextran matrix with the mAb, which is a highly basic protein with a p*I* of 9.4, and (ii) an electrostatic repulsion between the negatively charged antigen and the carboxymethyldextran matrix. Importantly, simple diagnostic tests can be performed early in the measurement process to identify these types of matrix-mediated artifacts.

The development of therapeutic monoclonal antibodies (mAbs)[5] requires rigorous measurements of the kinetic and thermodynamic binding properties of antigen/antibody (Ag/mAb) complexes. Accurate and precise measurements for the association rate constant (k_a), dissociation rate constant (k_d), and equilibrium dissociation constant or affinity (K_D) provide vital information informing the drug development process. The K_D may affect the efficacy of the mAb, influence the pharmacokinetics and dosing strategy, and/or influence the final drug cost given that tighter binding mAbs usually enable lower doses.

Ag/mAb equilibrium dissociation constants are best measured by surface plasmon resonance (SPR) biosensors and the solution-based kinetic exclusion assay (KinExA) [1–11]. In the study presented here, we chose the Biacore biosensor and immobilized or captured mAb to a biosensor surface while the Ag was flowed across the mAb surface. The Biacore instrument detects binding in real time by following the change in SPR as Ag binds to the mAb surface. By nonlinear fitting of the data, the k_d and k_a can be determined and then the K_D is calculated from the quotient of k_d/k_a.

The KinExA instrument is essentially a flow spectrofluorimeter where equilibrated Ag/mAb solutions are flowed through an Ag-coated bead pack. A small portion of the free mAb present in the equilibrated solution is bound to the bead pack, after which the adsorbed free mAb is detected by a fluorescently labeled, species-specific polyclonal antibody (pAb) [7,8]. KinExA technology is also able to measure the k_a directly by following the decrease in free mAb as a function of time as an Ag/mAb solution approaches equilibrium. The k_d cannot be determined directly with KinExA; rather, it is calculated by the product of $K_D \times k_a$. Usually, similar k_a, k_d, and K_D values are determined with these two platforms when experiments are designed correctly, the instrumentation is used properly,

* Corresponding author. Fax: +1 888 887 7210.
E-mail address: sklakamp@yahoo.com (S.L. Klakamp).
[1] Current address: Compugen Inc., 260 E. Grand Ave., South San Francisco, CA 94080, USA.
[2] Current address: Gilead Sciences Inc., 333 Lakeside Drive, Foster City, CA 94404, USA.
[3] Current address: DNA Bridges, Inc., 55 New Montgomery St., Suite 605, San Francisco, CA 94105, USA.
[4] Current address: SK_D Consulting LLC, 4119 Coriander Terrace, Fremont, CA 94538, USA.
[5] *Abbreviations used:* mAb, monoclonal antibody; Ag/mAb, antigen/antibody; SPR, surface plasmon resonance; KinExA, kinetic exclusion assay; pAb, polyclonal antibody; CM, carboxymethyl; IgG_1, immunoglobulin G_1; CHO, Chinese hamster ovary; BSA, bovine serum albumin; EDC, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide; NHS, *N*-hydroxysuccinimide; PBS, phosphate-buffered saline; RU, resonance units; LCM, least common multiplier.

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http://dx.doi.org/10.1016/j.ab.2012.06.024

and the resulting data are processed optimally [1,4,5,12,13]. However, on rare occasions, even well-performed Biacore and KinExA Ag/mAb measurements do not agree. Although these exceptions are infrequent, the measurement of an inaccurate affinity or binding kinetic profile can have a highly detrimental effect on the development of a therapeutic mAb.

In preliminary Biacore studies of this Ag/mAb interaction, the binding kinetics varied depending on the specific type of sensor chip used. To understand this unexpected result, a systematic, statistically powered study encompassing 53 Biacore and KinExA experiments was performed. Employing different carboxymethyldextran (CM–dextran) matrix-coated sensor chips with varying amounts of negative charge, we were able to demonstrate that results from experiments using the lower negative charge on the sensor surface were similar to the results derived from the solution-phase Biacore and KinExA experiments. During the course of the study, we devised three diagnostic tests that can be employed early in the Biacore measurement process to determine whether the CM–dextran matrix is introducing artifacts into the binding kinetics and affinity of an Ag/mAb interaction.

Materials and methods

Instrumentation

SPR experiments were performed using Biacore 2000 and Biacore T100 optical biosensors (GE Healthcare, Piscataway, NJ, USA). All KinExA experiments were performed using KinExA 3000 and KinExA 3200 instruments (Sapidyne Instruments, Boise, ID, USA).

Reagents

The ectodomain of the Ag is a monomer (predicted MW from amino acid sequence is 47,600; glycosylated MW is 81,000) and was produced and purified in-house. The mAb, immunoglobulin G_1 (IgG_1), was fully human and was expressed using a clonal Chinese hamster ovary (CHO) cell line from a recombinant proprietary CHO-based expression system at Millennium Pharmaceuticals. The stock concentrations of the Ag and the mAb were determined using the methods detailed by Grimsley and Pace [14]. The active concentration of Ag was calculated by multiplying the stock concentration by the average of the least common multiplier calculated from several KinExA equilibrium experiments (see below). It is well known that the measured protein concentration is often different from the active protein concentration. Although we used KinExA titrations to determine active Ag concentrations in this study, alternatively Ag activity could have been determined from partial mass transport limited Biacore data [15–17].

All Ag and mAb samples for Biacore and KinExA analyses were prepared in vacuum-degassed HBS-P buffer (0.01 M Hepes, 0.15 M NaCl, and 0.005% surfactant P-20) from GE Healthcare with 100 μg/ml filtered bovine serum albumin (BSA) and 0.02% sodium azide (Fisher Scientific, BP1605-100, Fair Lawn, NJ, USA). Biacore amine-coupling reagents, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC), *N*-hydroxysuccinimide (NHS), ethanolamine, and sodium acetate buffers (10 mM, pH 4.0 or pH 5.0) were purchased from GE Healthcare. Biacore aldehyde-coupling reagents, sodium periodate, carbohydrazide, and sodium cyanoborohydride were purchased from Sigma–Aldrich (St. Louis, MO, USA). Goat anti-human IgG pAb for Biacore capture experiments was purchased from Invitrogen (H10500, Camarillo, CA, USA). Biacore surface regeneration reagents were phosphoric acid from Fisher Scientific (A260-500, Fairfield, NJ, USA) for capture experiments and 10 mM glycine (pH 2.0, GE Healthcare) for covalently coupled mAb surfaces. Research-grade CM5, CM4, and C1 biosensor chips were purchased from GE Healthcare.

The detection antibody used for all KinExA experiments was Cy5-labeled goat anti-human IgG, Fcγ specific (Jackson ImmunoResearch Laboratories, West Grove, PA, USA), diluted 500- to 4000-fold in the HBS-P buffer (with BSA and sodium azide) described above from a 0.5-mg/ml stock in 1× phosphate-buffered saline (PBS, pH 7.4). The solid-phase particles used for all KinExA experiments were azlactone beads (Ultralink Support, Thermo Scientific, Rockford, IL, USA). For each KinExA experiment, approximately 10 μg of Ag diluted into 1 ml of 50 mM sodium carbonate (pH 9.2) was added directly to 50 mg of azlactone beads and either rocked overnight at 4 °C or rocked for at least 4 h at room temperature. After rocking, the beads were rinsed once with 1 M Tris buffer (pH 8.5) containing 10 mg/ml BSA and rocked for 1 h at room temperature in the same buffer. Coupled beads were diluted to approximately 30 ml with 1× HBS-N buffer (0.01 M Hepes [pH 7.4] and 0.15 M NaCl, GE Healthcare) containing 0.02% sodium azide in the KinExA bead reservoir and were used immediately.

Biacore kinetic measurements

Standard EDC/NHS coupling was used to covalently immobilize the therapeutic mAb to CM5, CM4, and C1 sensor chips. Several replicate experiments used aldehyde coupling to covalently immobilize mAb to a CM4 chip [18]. CM5 chips were activated with EDC/NHS for 7 min with excess activated carboxyl groups blocked with ethanolamine for 7 min following immobilization of mAb (diluted to 20 μg/ml in 10 mM sodium acetate, pH 4.0). CM4 and C1 chips were activated and blocked for 5 min. MAb was immobilized at levels that gave a maximum binding response (R_{max}) lower than 80 resonance units (RU) to minimize any potential mass transport and crowding artifacts. For all experiments with covalently immobilized mAb, one flow cell served as a reference surface following activation and blocking on each chip in the absence of mAb immobilization.

For capture experiments, high-density surfaces of goat anti-human pAb were prepared over all four flow cells of CM5, CM4, and C1 sensor chips using standard EDC/NHS coupling. CM5 chips were activated with EDC/NHS for 7 min with excess activated carboxyl groups blocked with ethanolamine for 7 min following pAb coupling. CM4 and C1 chips were activated and blocked for 5 min. For all three types of Biacore chips, anti-human pAb was diluted to 25 μg/ml in 10 mM sodium acetate (pH 5.0) and injected over all four activated flow cells for 30 min at a flow rate of 10 μl/min. For each capture experiment, the mAb drug was captured over three flow cells at a flow rate of 10 μl/min for 30 s, whereas one flow cell of the biosensor chip was left without captured mAb to provide a reference surface. The mAb was captured at a concentration of 0.9 to 1.7 μg/ml diluted in HBS-P buffer with 100 μg/ml BSA and 0.02% sodium azide. After capturing the mAb, running buffer was flowed over all flow cells for 120 s at a flow rate of 100 μl/min to stabilize the capture surfaces. The capture protocol was designed to yield capture levels of mAb that resulted in an R_{max} no greater than 80 RU.

For each Biacore kinetic experiment, a series of six or seven Ag concentrations serially diluted 2-fold was prepared in the running buffer and injected in triplicate in a random order for 2 min at 100 μl/min (CM4 and C1 chips) or for 4 min at 50 μl/min (CM5 chips) followed by 15 min of dissociation data. To obtain more extensive off-rate decay data, each experiment included three additional injections of the highest or second highest antigen concentration alternated with three additional buffer injections followed by a dissociation phase of 1 h. All covalent surfaces were regenerated with one 15-s pulse of 10 mM glycine–HCl (pH 2.0, GE Healthcare). Regeneration conditions were determined using the

Drake–Klakamp method [19]. All capture surfaces were regenerated with two 15-s pulses of 146 mM phosphoric acid. All Biacore kinetic experiments were conducted at 22 °C.

Biacore sensorgrams were processed using Scrubber software (version 2.0, BioLogic Software, Campbell, Australia). Sensorgrams were first zeroed on the *y* axis and then *x*-aligned at the beginning of the Ag injection. Bulk refractive index changes were removed by subtracting the responses from the designated reference flow cell. The average response of all blank injections was subtracted from all Ag and blank sensorgrams to remove systematic artifacts in the experimental and reference flow cells.

CLAMP 2000 biosensor data analysis software (version 3.40, BioLogic Software) was used to estimate k_a and k_d from the processed data sets. Data from the three experimental flow cells of a single biosensor chip were globally fit to a 1:1 bimolecular binding model that included a term for mass transport. The K_D was calculated from the quotient of k_d/k_a. The results from globally fitting data from three flow cells of a single biosensor chip were considered to be a single replicate measurement of the binding constants. Three to six independent replicate measurements (9–18 flow cells of data) were determined for both covalent and capture experiments using multiple CM5, CM4, and C1 biosensor chips.

KinExA equilibrium measurements

All KinExA equilibrium experiments were conducted at room temperature (~22 °C). For each experiment, Ag was serially diluted into solutions having a constant mAb binding site concentration. For K_D-controlled experiments, the mAb binding site concentration was approximately equal to the K_D and the prepared samples were equilibrated at room temperature for at least 6 h. For antibody-controlled experiments, the mAb binding site concentration was held at approximately 20-fold above the K_D and the prepared samples were allowed to equilibrate at room temperature for at least 2 h. The sample and labeling antibody flow rates were 0.25 ml/min for all experiments. During a K_D-controlled experiment, each sample was drawn through the flow cell at a volume that varied from 3.0 to 8.0 ml. For antibody-controlled experiments, sample volumes were drawn through the flow cell at volumes that ranged from 0.75 to 1.0 ml. Each replicate K_D measurement included data from a K_D-controlled titration simultaneously fit with data from an antibody-controlled titration using a 1:1 equilibrium binding model with KinExA software (version 3.1.3, Sapidyne Instruments) using the "antigen unknown" dual-curve algorithm [8]. Four dual-curve experiments were performed using standard buffer conditions, whereas six dual-curve experiments were performed with 10 mg/ml CM–dextran–sodium salt (Sigma–Aldrich) added to all buffers.

KinExA kinetic measurements

The k_a of the Ag/mAb complex was measured using the KinExA "direct method" as described previously [1,8]. The resulting exponential function of free mAb binding site as a function of time was fit in the KinExA software to the analytical reversible bimolecular rate equation. To calculate the k_d value, each measured replicate of k_a was multiplied by the corresponding mean K_D value determined from several replicate KinExA K_D measurements of the Ag/mAb complex. Four "direct method" experiments were performed using standard buffer conditions, whereas five direct method experiments were performed with 10 mg/ml CM–dextran–sodium salt added to all buffers.

Biacore solution-phase equilibrium measurements

All Biacore solution-phase experiments were conducted at 22 °C. For each equilibrium measurement using Biacore, a high-density surface of the therapeutic mAb was prepared using standard EDC/NHS coupling chemistry to a CM5 chip. A reference surface was also prepared as described above. Samples were prepared by serially diluting the mAb into solutions having a constant concentration of Ag. For K_D-controlled experiments, the concentration of Ag was 2-fold above the K_D and the prepared samples were equilibrated for at least 6 h. For antigen-controlled experiments, the concentration of Ag was 21-fold above the K_D and the prepared samples were equilibrated for at least 2 h. Equilibrated Ag/mAb samples prepared for the K_D-controlled experiments were injected over both the experimental and reference flow cells for 50 min. Samples prepared for the antigen-controlled experiments were injected over both the experimental and reference flow cells for 25 min. All Ag/mAb samples were injected in triplicate at a flow rate of 5 μl/min with several buffer injections interspersed for double-referencing. Dissociation was followed for 2 min only to provide sensorgrams with a more apparent end point to the injection. Surface regeneration after each injection was a single 15-s pulse of 10 mM glycine–HCl (pH 2.0) over both flow cells.

The Biacore sensorgrams were processed as described for the Biacore kinetic measurements using Scrubber software. Binding responses to the surface resulting from unbound Ag remaining in each equilibrated solution were recorded at an identical time point for all sensorgrams, after double-referencing, using the "bound" feature in Scrubber. The response levels for both K_D-controlled and antigen-controlled titrations were plotted as a function of the total mAb binding site concentration for each sample using the KinExA "n-curve" data analysis software. The resulting dual titration curves were globally fit to a 1:1 equilibrium model using the KinExA software to estimate the K_D.

Results

KinExA measurements

Four independent dual-curve K_D measurements of the Ag/mAb complex were performed by titrating Ag (45.9 fM–2.35 nM) into 12 solutions of 26.9 pM mAb binding site, thereby holding the binding site concentration constant. Each mAb-controlled titration experiment was prepared by equilibrating 12 concentrations of Ag at a range of 81.1 fM to 4.15 nM with 437 pM mAb binding site. The resulting four dual-curve titrations were each globally fit to a 1:1 equilibrium model with drift correction.

The "standard" affinity model in the KinExA fitting software, which assumes 100% activity of the Ag and floats the binding site concentration of the mAb during the nonlinear fitting, returned a 95% confidence interval for the $[mAb]_{binding\ site}$ with a lower limit that was greater than the actual binding site concentration of mAb used in each titration. Because the mAb activity cannot exceed 100%, the Ag certainly was less than 100% active in this study. Therefore, we employed the "antigen unknown" fitting model available in the KinExA software package. This model assumes that the active proportion of the Ag is unknown [8] and uses a least common multiplier (LCM) value for all Ag concentrations, and the mAb is assumed to be 100% active. Essentially, the LCM value is the factor that adjusts the hard-coded Ag concentrations to their active concentrations. The dual-curve KinExA replicates (without soluble CM–dextran) analyzed using the "antigen unknown" algorithm yielded K_D = 22.1 (4.7 [95% confidence interval]) pM (Table 1). The average LCM measured was 0.564 (0.134), indicating that the Ag was 56.4% active. To maintain consistency throughout this biophysical comparison study, the active concentrations of Ag prepared for all KinExA kinetic measurements, Biacore kinetic measurements,

Table 1
Ag/mAb comparison results.

Method	*n*	k_a (M^{-1} s^{-1})	k_d (s^{-1})	K_D (pM)
Biacore				
CM5 (amine)	5	1.13 (0.04) × 10^5	2.23 (0.10) × 10^{-4}	1970 (140)
CM5 (capture)	3	5.81 (0.12) × 10^4	1.67 (0.05) × 10^{-4}	2870 (120)
CM4 (amine)	4	2.87 (0.16) × 10^5	1.91 (0.06) × 10^{-4}	664 (47)
CM4 (aldehyde)	4	3.19 (1.05) × 10^5	1.78 (0.05) × 10^{-4}	580 (219)
CM4 (capture)	3	1.28 (0.30) × 10^5	1.65 (0.14) × 10^{-4}	1290 (260)
C1 (amine)	6	1.02 (0.06) × 10^6	1.91 (0.09) × 10^{-4}	186 (8)
C1 (capture)	3	8.60 (0.47) × 10^5	2.86 (0.44) × 10^{-4}	333 (63)
Solution phase	6	n/a	n/a	91.9 (32.4)
KinExA				
Standard	4 K_D, 4 k_a	3.31 (0.10) × 10^6	7.32 (0.23) × 10^{-5}	22.1 (4.7)
With dextran	6 K_D, 5 k_a	2.45 (0.18) × 10^6	1.47 (0.10) × 10^{-4}	60.1 (27.3)

Note: k_a, association rate constant; k_d, dissociation rate constant; K_D, equilibrium dissociation constant; n/a, not applicable. The numbers shown in parentheses are the 95% confidence intervals.

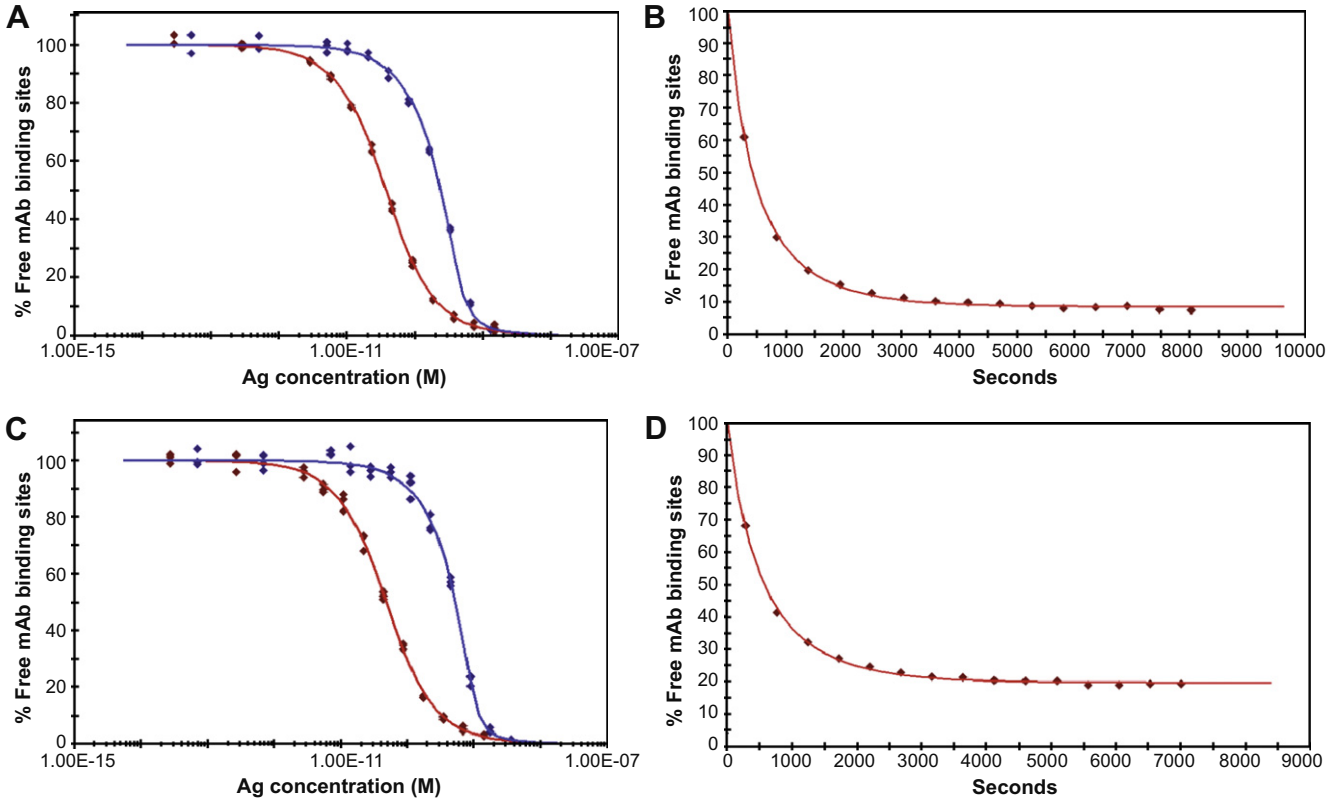

Fig.1. (A) Dual-curve KinExA equilibrium titration of the Ag/mAb complex. Triplicate data points were acquired for the K_D-controlled titration (fitted curve in red) where an Ag concentration range of 45.9 fM to 2.35 nM was titrated into 26.9 pM mAb binding site. Triplicate data points for the mAb-controlled titration (fitted curve in blue) were acquired by titrating an Ag concentration range of 81.0 fM to 4.15 nM into 437 pM mAb binding site. Both curves were simultaneously fit to a 1:1 equilibrium model using the "antigen unknown" method to estimate K_D and generate a least common multiplier (LCM) for the active concentration of Ag: K_D = 24.7 pM, LCM = 0.638. (B) KinExA "direct method" measurement of the k_a of the Ag/mAb complex. Each point indicating the percentage free mAb binding site was acquired every 9 min as 449 pM mAb binding sites and 655 pM Ag reacted in solution over 130 min: $k_a = 3.26 \times 10^6$ M^{-1} s^{-1}. (C) Dual-curve KinExA data of the Ag/mAb complex obtained with 10 mg/ml CM–dextran added to all buffers. For the K_D-controlled titration (fitted curve in red), an Ag concentration range of 53.9 fM to 2.76 nM was titrated into 30.3 pM mAb binding site. The mAb-controlled titration (fitted curve in blue) was acquired by titrating an Ag concentration range of 138 fM to 7.05 nM into 1.00 nM mAb binding site. Curves were simultaneously fit with the "antigen unknown" equilibrium model: K_D = 33.2 pM, LCM = 0.509. (D) KinExA "direct method" measuring the k_a of the Ag/mAb complex with 10 mg/ml CM–dextran added to all buffers. The percentage free mAb binding site was acquired every 9 min as 502 pM mAb binding site and 649 pM Ag reacted in solution over 116 min: $k_a = 2.66 \times 10^6$ M^{-1} s^{-1}.

and Biacore solution-phase K_D measurements were adjusted with this LCM factor when incorporated into their respective fitting algorithms. A representative equilibrium KinExA data set under standard buffer conditions is shown in Fig. 1A.

The k_a was determined by the KinExA "direct method" where solutions (without CM–dextran added) containing 449 pM mAb binding site and 655 pM Ag were followed to equilibrium for each replicate measurement. Free mAb binding site was quantified as a function of time after mixing Ag with mAb. A 1:1 kinetic monoexponential function was used to fit the data and ultimately determine the k_a. Four independent KinExA kinetic experiments yielded k_a = 3.31 (0.10) × 10^6 M^{-1} s^{-1}. Each independent measurement of k_a was multiplied by the average K_D value (22.1 pM) obtained from the four corresponding equilibrium experiments

to yield an average $k_d = 7.32\ (0.23) \times 10^{-5}\ s^{-1}$. A typical KinExA experiment for the determination of k_a under standard buffer conditions is shown in Fig. 1B.

To mimic the Biacore dextran environment in which protein/protein interactions take place, six independent dual-curve KinExA equilibrium titrations were performed with 10 mg/ml soluble CM–dextran added to all samples and all buffers. Every K_D-controlled titration experiment was performed with 30.3 pM mAb binding site equilibrated with an Ag concentration range of 53.9 fM to 2.76 nM. Two mAb-controlled titration experiments were performed with 504 pM mAb binding site equilibrated with an Ag concentration range of 97.0 fM to 4.97 nM, and four mAb-controlled experiments were performed with 1.00 nM mAb binding site equilibrated with Ag at 138 fM to 7.05 nM. The six dual-curve titrations were fit using the "antigen unknown" equilibrium model, which yielded a $K_D = 60.1\ (27.3)$ pM. For these equilibrium experiments, the [Ag] used was the hard-coded concentration because the "antigen unknown" algorithm was used for fitting. The average LCM measured was 0.652 (0.184), which overlapped with the LCM measured in the absence of CM–dextran.

Five independent association kinetic measurements were made using the "direct method" in the presence of soluble dextran. Concentrations of Ag and mAb were identical to the kinetic experiments performed with standard buffer conditions. The independent measurements yielded a $k_a = 2.45\ (0.18) \times 10^6\ M^{-1}\ s^{-1}$. When each measurement of k_a was multiplied by 60.1 pM, the resulting average $k_d = 1.47\ (0.10) \times 10^{-4}\ s^{-1}$. Representative data sets for K_D and k_a measurements in the presence of soluble dextran are shown in Fig. 1C and D, respectively.

Biacore kinetic measurements

A CM5 Biacore chip is generally considered to be the standard biosensor chip for Biacore experiments. A CM4 Biacore chip contains approximately 30% of the carboxyl groups and a C1 chip has approximately 10% of the carboxyl groups when both are compared with a CM5 chip [18]. On C1 chips, a mercapto–alkane–carboxyl linker is bonded directly to the gold surface of the biosensor and no dextran matrix is present on the surface as with CM4 and CM5 chips. Several replicate kinetic measurements of the Ag/mAb complex studied here were performed with all three chip variations where mAb was immobilized to the surface both covalently and with capture methods. This particular experimental orientation with mAb immobilized or captured to the surface was crucial to avoid avidity (cross-linking) effects if mAb had been injected over Ag immobilized to the surface. The orientation we used was desirable for three reasons: (i) avid binding to a biosensor surface most often results in complex kinetics that cannot be fit with a simple 1:1 kinetic interaction model; (ii) as Ag surface densities change, the amount of avid binding of the mAb to the surface could change, resulting in inconsistent measurements for k_a and k_d; and (iii) even if the sensorgrams from flowing mAb over Ag could be fit with a 1:1 interaction model, it would be unknown whether what was being measured was the intrinsic site binding constants for the Ag/mAb complex or whether avidity constants were being measured, the latter being inconsistent with the site binding constants being measured using KinExA and solution-based Biacore experiments in this study [20–22]. The double-referenced sensorgrams were highly reproducible for each data set. They were globally fit to a 1:1 kinetic model with a term for mass transport, and each gave an excellent fit to a 1:1 kinetic interaction model.

When mAb was covalently immobilized to a CM5 chip using amine coupling, it was necessary for the mAb immobilization levels to range unexpectedly from 2431 to 5783 RU over three flow cells of each CM5 chip to achieve R_{max} values for Ag binding that ranged from 8 to 75 RU (see Discussion). Ag samples were injected at a concentration range of 1.82 to 58.3 nM. The dissociation phase of three additional injections of Ag at 58.3 nM was followed for 1 h. Fig. 2A shows a representative data set where mAb was amine coupled to a CM5 chip. Five independent experiments, which used amine coupling with five different CM5 chips totaling 15 flow cells with covalently immobilized mAb, yielded an average $k_a = 1.13\ (0.04) \times 10^5\ M^{-1}\ s^{-1}$, an average $k_d = 2.23\ (0.10) \times 10^{-4}\ s^{-1}$, and a calculated $K_D = 1.97\ (0.14)$ nM.

For capture experiments using CM5 chips, anti-human pAb was amine coupled at levels that ranged from 7477 to 8504 RU. Capture levels of mAb varied from 80 to 200 RU, giving R_{max} values for Ag binding that ranged from 26 to 54 RU. Concentrations of Ag from 1.82 to 117 nM were injected. A longer dissociation phase was followed for three additional injections of Ag at 58.3 nM. Fig. 2B shows a replicate data set of a capture experiment using a CM5 chip. Three independent capture experiments totaling 9 flow cells on three different CM5 chips yielded an average $k_a = 5.81\ (0.12) \times 10^4\ M^{-1}\ s^{-1}$, $k_d = 1.67\ (0.05) \times 10^{-4}\ s^{-1}$, and $K_D = 2.87\ (0.12)$ nM.

MAb also was covalently immobilized to several CM4 chips using both amine-coupling and aldehyde-coupling chemistries and to C1 chips using only amine-coupling methods. MAb was amine coupled to CM4 chips with immobilization levels ranging from 127 to 477 RU that resulted in R_{max} values between 11 and 43 RU. When mAb was aldehyde coupled to the surface of CM4 chips, R_{max} values of 15 to 30 RU resulted from immobilization levels that ranged from 107 to 160 RU. MAb was amine coupled on C1 chips to 178 to 259 RU, which gave rise to R_{max} values of 20 to 37 RU. Ag samples were injected at a concentration range of 1.10 to 70.1 nM for experiments where mAb was covalently coupled to CM4 chips and at a concentration range of 1.10 to 35.0 nM for all amine-coupled C1 chips. Longer dissociation sensorgram data for three additional injections of Ag at 35.0 nM were followed with all CM4 and C1 chips having covalently coupled mAb. Fig. 3A and B show representative data sets with mAb amine coupled and aldehyde coupled to a CM4 biosensor chip, respectively. Fig. 4A illustrates a representative experiment with mAb amine coupled to a C1 chip. Four independent experiments with mAb amine coupled to a total of 12 flow cells over four different CM4 chips resulted in an average $k_a = 2.87\ (0.16) \times 10^5\ M^{-1}\ s^{-1}$, $k_d = 1.91\ (0.06) \times 10^{-4}\ s^{-1}$, and $K_D = 664\ (47)$ pM. The same experimental rigor was applied with aldehyde coupling to yield an average $k_a = 3.19\ (1.05) \times 10^5\ M^{-1}\ s^{-1}$, $k_d = 1.78\ (0.05) \times 10^{-4}\ s^{-1}$, and $K_D = 580\ (219)$ pM. The Biacore results from six independent experiments with six different amine-coupled C1 chips (18 total flow cells) gave an average $k_a = 1.02\ (0.06) \times 10^6\ M^{-1}\ s^{-1}$, $k_d = 1.91\ (0.09) \times 10^{-4}\ s^{-1}$, and $K_D = 186\ (8)$ pM.

In addition, capture experiments were performed using CM4 and C1 biosensor chips. Anti-human pAb was amine coupled to CM4 chips at a range of 1350 to 1750 RU, whereas capture levels of mAb ranged from 60 to 120 RU, resulting in R_{max} values of 30 to 50 RU. The covalent immobilization range of pAb with C1 chips was 194 to 428 RU with mAb captured at a range of 30 to 120 RU and yielding R_{max} values of 21 to 77 RU. Ag was injected at a concentration range of 1.82 to 117 nM with CM4 chips and 0.913 to 58.4 nM with C1 chips. Additional Ag samples of 58.3 and 29.2 nM were injected over CM4 and C1 chips, respectively, to acquire 1-h dissociation data. Fig. 3C and Fig. 4B show replicate capture experiments using CM4 and C1 chips, respectively. Three independent capture experiments using three different CM4 chips (9 flow cells) yielded an average $k_a = 1.28\ (0.30) \times 10^5\ M^{-1}\ s^{-1}$, $k_d = 1.65\ (0.14) \times 10^{-4}\ s^{-1}$, and $K_D = 1.29\ (0.26)$ nM. With the same experimental format, employing the C1 chips yielded $k_a = 8.60\ (0.47) \times 10^5\ M^{-1}\ s^{-1}$, $k_d = 2.86\ (0.44) \times 10^{-4}\ s^{-1}$, and $K_D = 333\ (63)$ pM.

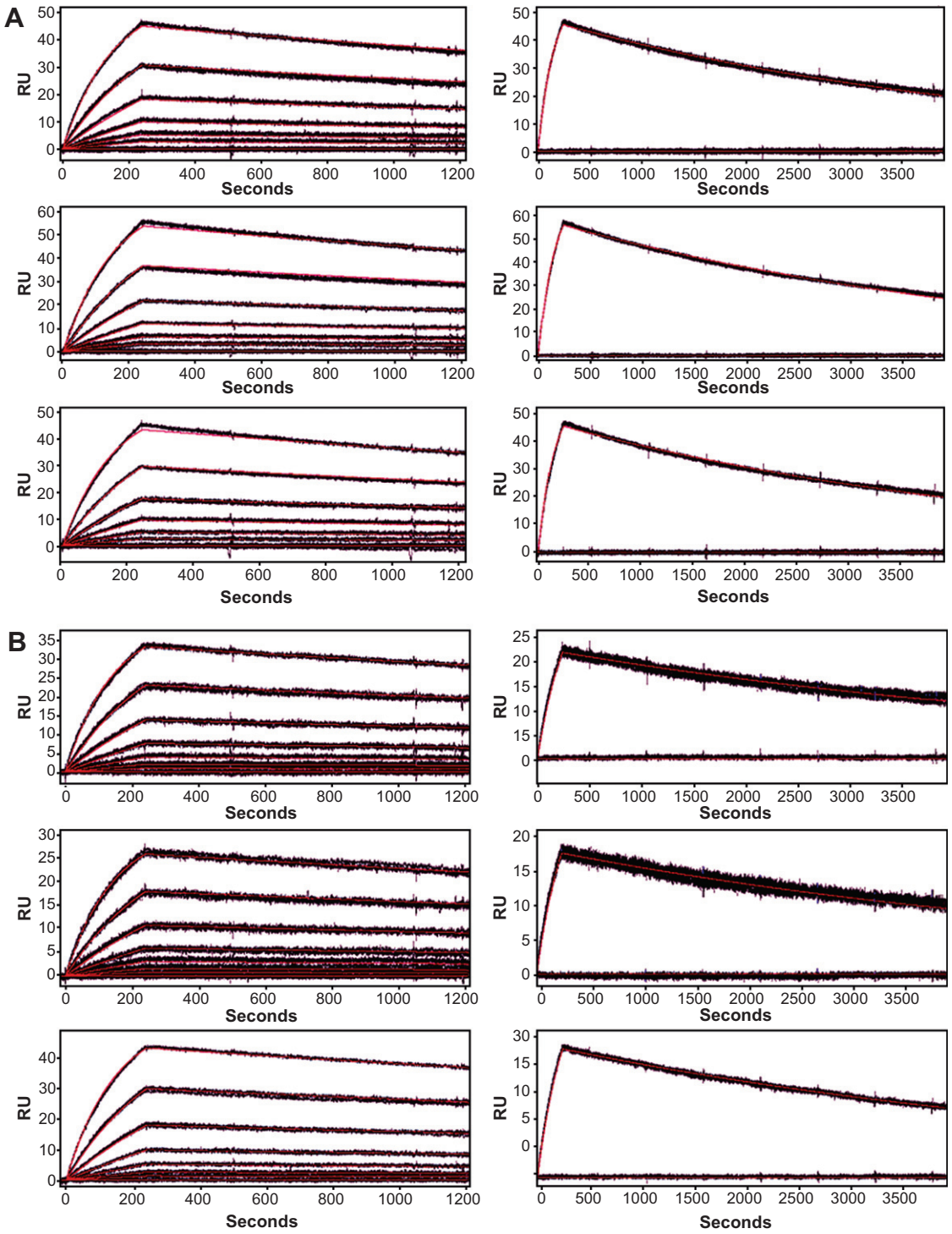

Fig.2. (A) Biacore data of Ag binding to mAb amine coupled to a CM5 chip. Panels on the left show double-referenced sensorgrams from a series of six Ag concentrations (1.82–58.3 nM) injected for 4 min with dissociation followed for 15 min. Panels on the right show additional triplicate injections of the highest concentration of Ag (58.3 nM) with dissociation followed for 1 h. Each black line in both sets of panels is actually three overlaid sensorgrams. All data were globally fit (red lines) over three independent flow cells: $k_a = 1.10 \times 10^5\ M^{-1}\ s^{-1}$; $k_d = 2.26 \times 10^{-4}\ s^{-1}$; $K_D = 2.05$ nM. (B) Biacore data of Ag binding to mAb captured to a CM5 Biacore chip. Panels on the left show double-referenced sensorgrams from a series of seven Ag concentrations (1.82–117 nM) injected for 4 min with dissociation followed for 15 min. Panels on the right show repeat injections of the second highest concentration of Ag (58.3 nM) with dissociation followed for 1 h. Each black line in both sets of panels is three overlaid sensorgrams. All data were globally fit (red lines) over three independent flow cells: $k_a = 5.83 \times 10^4\ M^{-1}\ s^{-1}$; $k_d = 1.67 \times 10^{-4}\ s^{-1}$; $K_D = 2.86$ nM.

Biacore solution-phase equilibrium measurements

To measure the K_D of the Ag/mAb complex using a Biacore method independent of binding kinetics, a solution-phase Biacore experiment, often incorrectly referred to as a "Biacore solution competition experiment", was performed [23]. This type of experiment is essentially the Biacore analog to a KinExA equilibrium experiment. The Biacore solution-phase affinity method uses a biosensor chip to capture unbound protein in equilibrated solutions of two binding partners; thus, the biosensor chip acts similarly to the

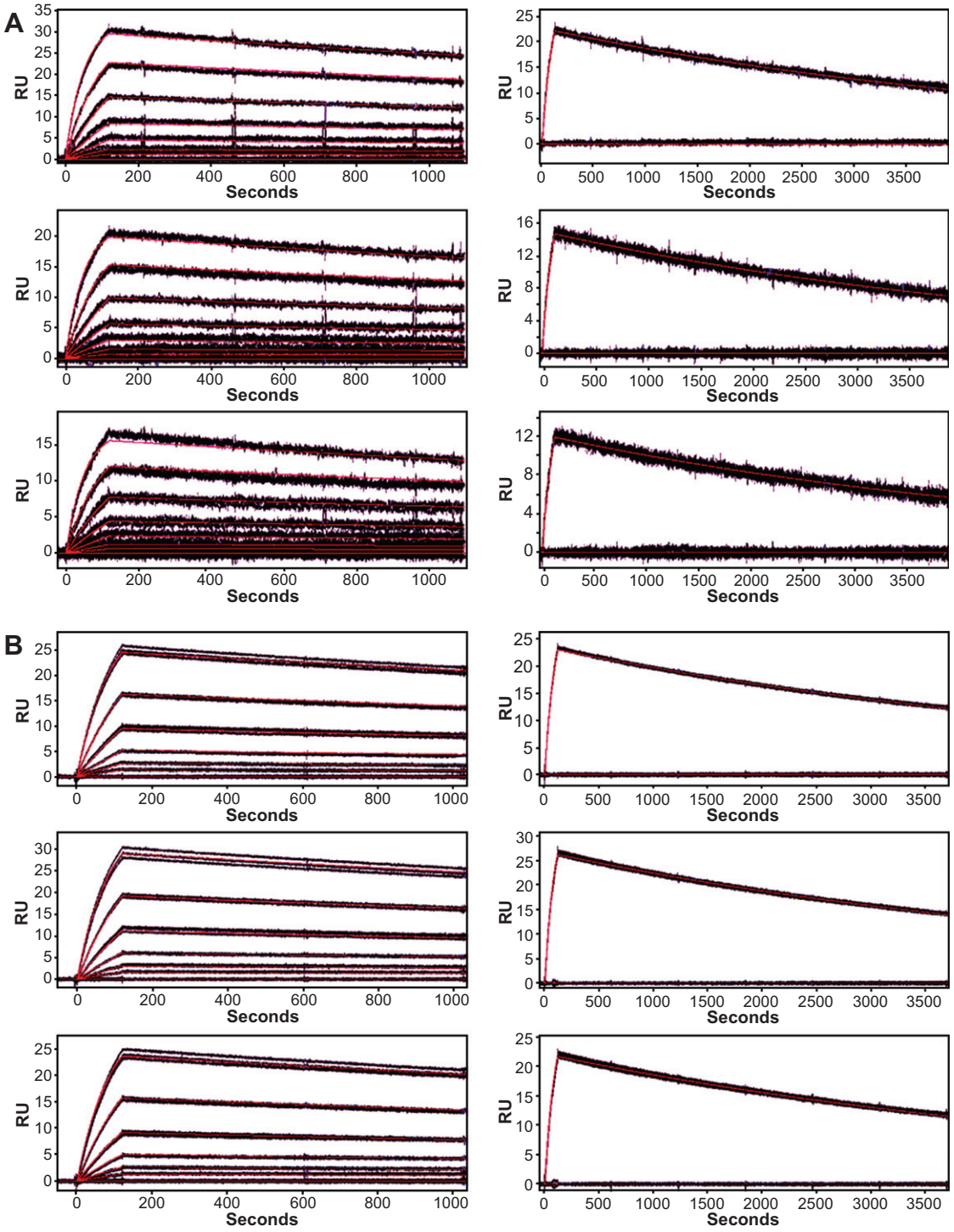

Fig.3. (A) Biacore data of Ag binding to mAb amine coupled to a CM4 chip. Panels on the left show double-referenced sensorgrams from a series of seven Ag concentrations (1.10–70.1 nM) injected for 2 min with dissociation followed for 15 min. Panels on the right show repeat injections of the second highest concentration of Ag (35.0 nM) with dissociation followed for 1 h. Each black line in both sets of panels is three overlaid sensorgrams. All data were globally fit (red lines) over three independent flow cells: $k_a = 2.90 \times 10^5\ M^{-1}\ s^{-1}$; $k_d = 1.95 \times 10^{-4}\ s^{-1}$; $K_D = 672$ pM. (B) Biacore data of Ag binding to mAb aldehyde coupled to a CM4 Biacore chip. Panels on the left show double-referenced sensorgrams from a series of six Ag concentrations (1.10–35.0 nM) injected for 2 min with dissociation followed for 15 min. Panels on the right show repeat injections of the highest concentration of Ag (35.0 nM) with dissociation followed for 1 h. All data were globally fit (red lines) over three independent flow cells: $k_a = 3.26 \times 10^5\ M^{-1}\ s^{-1}$; $k_d = 1.76 \times 10^{-4}\ s^{-1}$; $K_D = 540$ pM. (C) Biacore data of Ag binding to mAb captured to a CM4 chip. Panels on the left show double-referenced sensorgrams from a series of seven Ag concentrations (1.82–117 nM) injected for 2 min with dissociation followed for 15 min. Panels on the right show repeat injections of the second highest concentration of Ag (58.3 nM) with dissociation followed for 1 h. All data were globally fit (red lines) over three independent flow cells: $k_a = 1.34 \times 10^5\ M^{-1}\ s^{-1}$; $k_d = 1.61 \times 10^{-4}\ s^{-1}$; $K_D = 1.20$ nM.

bead pack used in KinExA experiments. In this study, the Biacore solution-phase protocol consisted of preparing equilibrated solutions of mAb titrated into a constant concentration of Ag. After equilibration, these solutions were injected over a single high-density surface of covalently immobilized mAb (amine coupled at immobilization levels of 15,585–17,821 RU) and a reference

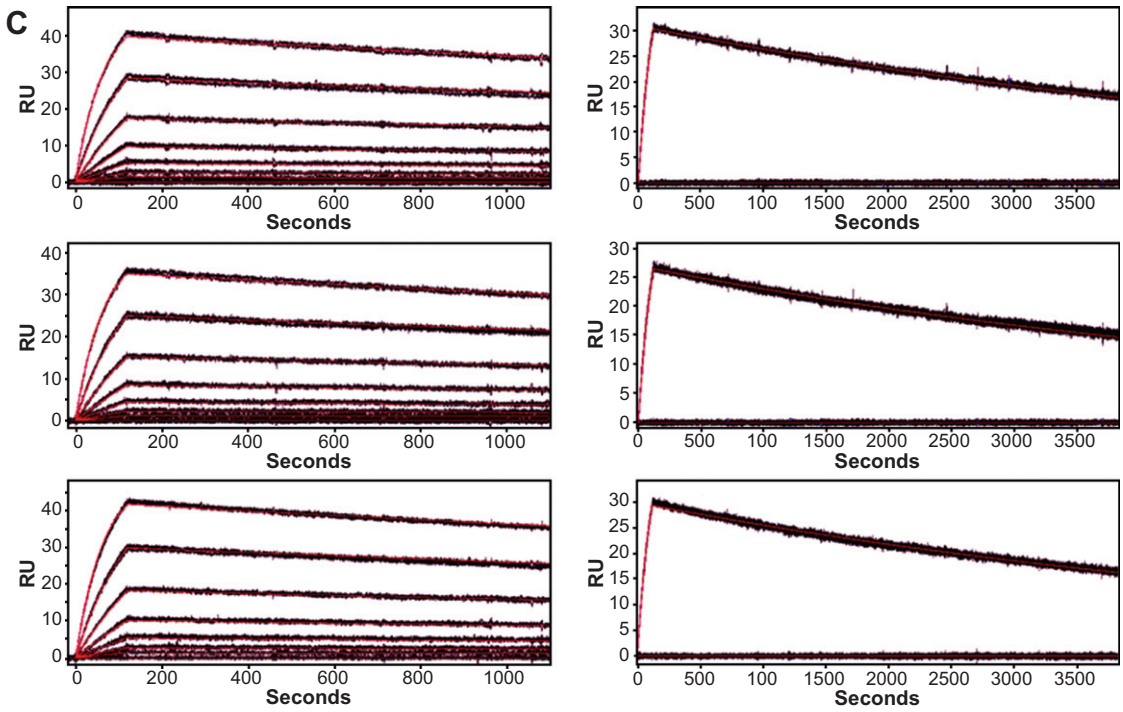

Fig. 3 (*continued*)

surface on a CM5 Biacore chip to determine the amount of unbound Ag in each equilibrated solution. In this format, the amount of unbound Ag in each solution was proportional to the Biacore binding responses such that the resulting sensorgrams exhibited a linear response [15–17,24]. In contrast to conventional Biacore solution-phase experiments that use a calibration curve to correlate binding responses to free protein concentrations, for the Biacore solution-phase experiments conducted here, the KinExA software was used to determine the K_D. For this application, only a signal that is proportional to the free concentration of the protein in the equilibrated solution is required in the KinExA software; an absolute value for the free protein concentration is not required for analysis. For each dual-curve analysis using this solution-phase Biacore protocol, the K_D-controlled samples were prepared with 12 concentrations of mAb binding site ranging from 37.9 fM to 1.94 nM equilibrated with 173 pM Ag, whereas all "antigen-controlled" samples had 12 concentrations of mAb binding site ranging from 191 fM to 9.78 nM equilibrated with a constant Ag concentration of 1.97 nM. Biacore response levels for the equilibrated solutions having the highest concentrations of free Ag ranged from 21 to 58 RU for K_D-controlled samples and from 117 to 173 RU for antigen-controlled samples. A 1:1 equilibrium fitting model ("standard" affinity model) with drift correction in the KinExA "n-curve analysis" software was used to estimate the K_D from each dual-curve experiment. The calculation of K_D was based on the concentration of mAb binding site in these experiments. This concentration basis is consistent with all of the previously described Biacore and KinExA experiments in this article. Six independent Biacore dual-curve titrations, each with a different CM5 chip, yielded a $K_D = 91.9$ (32.4) pM. A representative dual-curve titration is shown in Fig. 5.

Discussion

Here we have demonstrated that the binding kinetics and the K_D for an Ag/mAb interaction are adversely affected by the CM–dextran matrix found on most Biacore chips. Clearly, the mAb or the Ag is influenced by the surface negative charge in the CM–dextran layer (Fig. 6). The therapeutic mAb drug in this study is an unusual mAb in terms of its basicity with a p*I* of 9.4 (unpublished data). One hypothesis for the CM–dextran matrix effect is that an electrostatic interaction between the highly positively charged mAb and the negatively charged CM–dextran layer interferes, or at least competes, with the binding of mAb to the Ag. In contrast, the Ag is an acidic protein with ectodomain isoforms having measured pI values ranging from 5.0 to 6.5 (unpublished data). An alternative hypothesis for the CM–dextran matrix effect is that the k_a of the negatively charged Ag to mAb is slowed in the CM–dextran matrix by electrostatic repulsion.

To demonstrate that the CM–dextran layer was influencing the binding kinetics and affinity of the Ag/mAb complex, Biacore studies were conducted by using CM5, CM4, and C1 sensor chips, which have decreasing amounts of negatively charged carboxyl groups on their respective surfaces. The Biacore CM5 chip is the standard sensor surface used for protein analysis. The absolute concentration of carboxyl groups contained per unit volume in the dextran surface layer is proprietary information (personal communication, GE Healthcare). However, an estimate of this value can be calculated from published information, as shown in the Supplementary material [18,25,26]. The CM4 chip contains approximately 30% of the carboxyl groups in an identical dextran surface layer to a CM5 chip, whereas the C1 chip has no dextran layer at all but contains 10% of the carboxyl groups relative to a CM5 chip [18]. The carboxyl groups on the C1 gold surface are attached by a mercapto–alkane–carboxyl linker that forms a monolayer on the gold surface.

Table 1 illustrates convincingly that as the negative charge decreases on the biosensor surface, the measured kinetic rate constants and equilibrium dissociation constants approach the kinetic rate and binding constants observed in Biacore solution-phase and KinExA experiments. The first indication that the surface matrix may have been interfering with the binding of the Ag to the mAb came from the observation that approximately 2400 to 5800 RU of mAb needed to be immobilized by amine coupling to a CM5

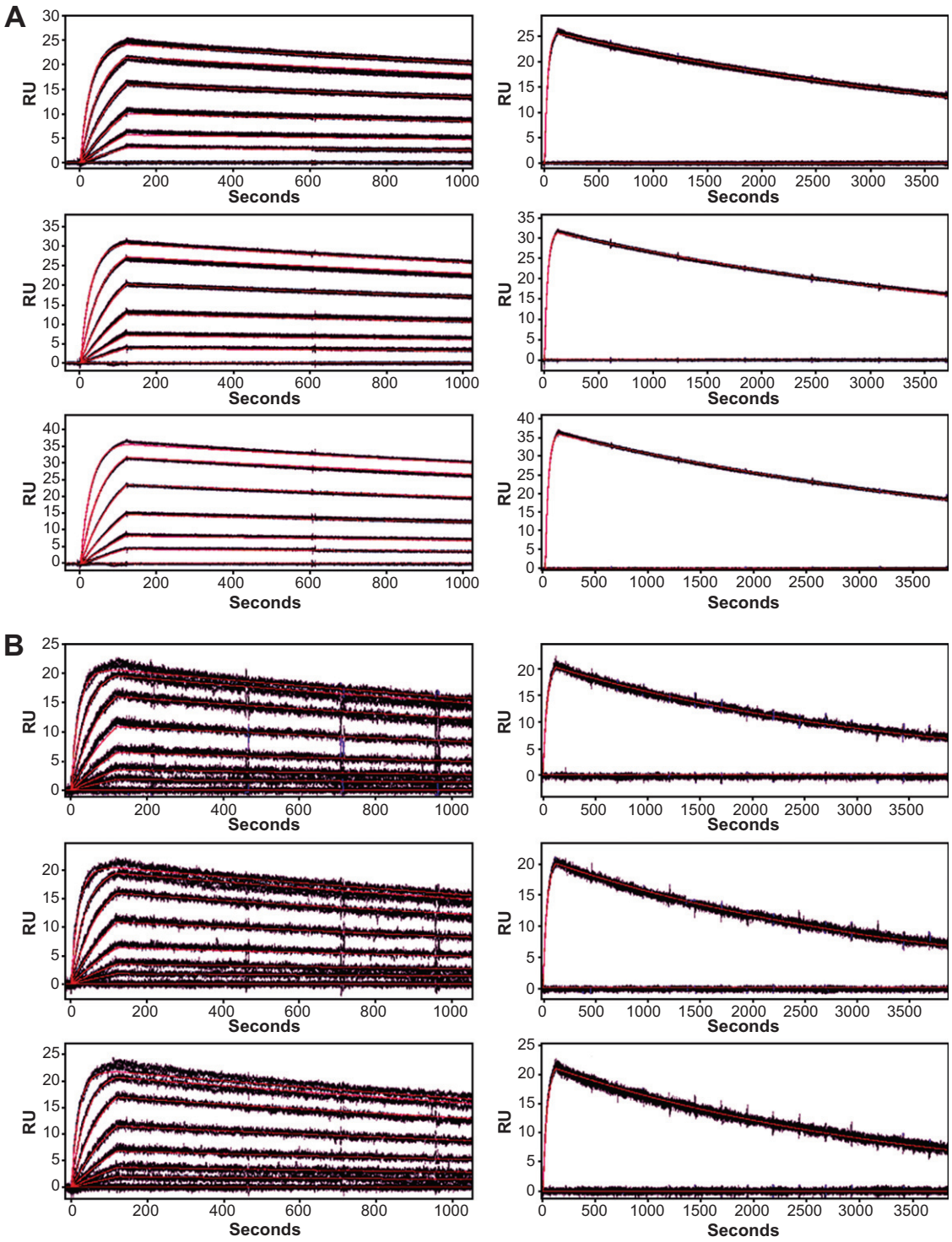

Fig.4. (A) Biacore data of Ag binding to mAb amine coupled to a C1 chip. Panels on the left show double-referenced sensorgrams from a series of six Ag concentrations (1.10–35.0 nM) injected for 2 min with dissociation followed for 15 min. Panels on the right show repeat injections of the highest concentration of Ag (35.0 nM) with dissociation followed for 1 h. Each black line is three overlaid sensorgrams. All data were globally fit (red lines) over three independent flow cells: $k_a = 1.02 \times 10^6\ M^{-1}\ s^{-1}$; $k_d = 1.88 \times 10^{-4}\ s^{-1}$; $K_D = 184$ pM. (B) Biacore data of Ag binding to mAb captured to a C1 chip. Panels on the left show double-referenced sensorgrams from a series of seven Ag concentrations (0.913–58.4 nM) injected for 2 min with dissociation followed for 15 min. Panels on the right show repeat injections of the second highest concentration of Ag (29.2 nM) with dissociation followed for 1 h. All data were globally fit (red lines) over three independent flow cells: $k_a = 8.72 \times 10^5\ M^{-1}\ s^{-1}$; $k_d = 2.94 \times 10^{-4}\ s^{-1}$; $K_D = 337$ pM.

chip to observe an R_{max} of 8 to 75 RU. This R_{max} shortfall implied that the mAb was only 0.8% active on average, which is remarkably low for a mAb. Typically, 40% to 60% of a mAb remains active after covalent coupling to a biosensor surface. The mAb presented here contains 88 lysine residues compared with the usual number of 80 to 84 lysines for an IgG_1 mAb. It is tempting to attribute the low mAb activity observed with amine coupling to inactivation of lysine residues located in the complementary-determining regions

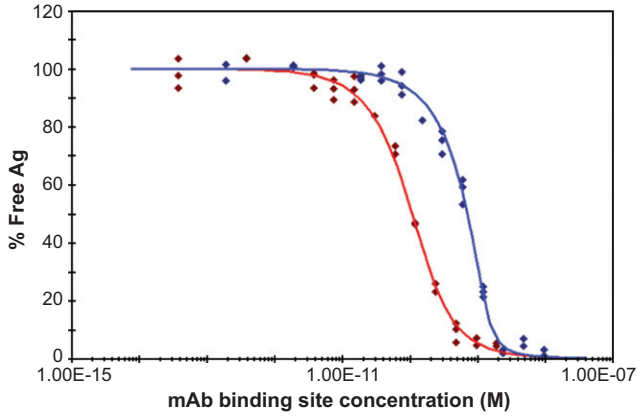

Fig.5. Dual-curve equilibrium titration data of the Ag/mAb complex obtained using a Biacore solution-phase method independent of binding kinetics. Free Ag in equilibrated solutions of Ag/mAb is detected with a flow cell containing a high-density surface of mAb. Triplicate data points were acquired for the K_D-controlled titration (red curve) where a mAb binding site concentration range of 37.9 fM to 1.94 nM was titrated into 173 pM Ag. For the antigen-controlled titration (blue curve), data were collected in triplicate with a constant [Ag] of 1.97 nM and [mAb binding site] ranging from 191 fM to 9.78 nM. Both curves were simultaneously fit to the "standard" affinity 1:1 equilibrium model using KinExA software to estimate the K_D at 50.9 pM.

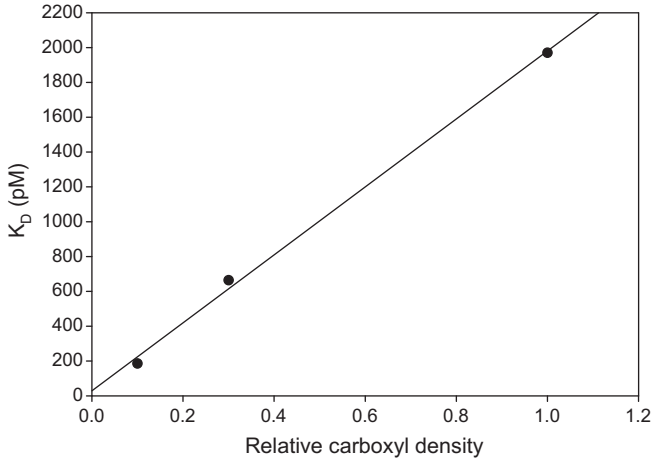

Fig.6. Correlations between the K_D and the relative negative charge of the dextran matrix for Ag binding to amine-coupled mAb on C1, CM4, and CM5 chips.

of the mAb. However, the values observed with amine and aldehyde coupling using CM4 and C1 chips suggest other possibilities for the low mAb activity measured on a CM5 chip. A plausible explanation for this low activity is that mAbs buried in the CM–dextran layer are inaccessible to bind with Ag due to an electrostatic interaction with the CM–dextran layer and, therefore, only mAbs near the top of the CM–dextran layer are able to interact with Ag. Alternatively, the Ag might not be able to bind to mAb binding sites buried deep with the dextran matrix owing to strong electrostatic repulsive forces between the negatively charged Ag and the CM–dextran matrix. In both of these explanations, we speculate that the CM–dextran is responsible for the low mAb activity observed.

A capture method also was employed on a CM5 chip. Here, 80 to 200 RU of mAb was captured by 7477 to 8504 RU of amine-coupled anti-human pAb on the CM5 surface. The observed R_{max} was approximately 26 to 54 RU, suggesting an average mAb activity of approximately 28%. Although markedly higher than with amine coupling, it was still lower than that usually observed with capture experiments with other mAbs. In capture format, mAb activity usually ranges from 50% to nearly 100%. As a general rule, it is dubious to consider kinetic measurements from a 0.8% active surface as representative of the kinetics that would normally be measured from a reasonably active biosensor surface. However, the amine-coupled and capture experiments produced similar binding kinetics and affinity measurements, indicative that the binding constants on the amine-coupled surface were correctly measured for a CM5 chip. Even though both immobilization methods yielded similar binding parameters from a CM5 chip, these proved to be inaccurate when compared with the Biacore solution-phase and KinExA results.

To further study the effects of surface negative charge on this Ag/mAb binding interaction, the binding measurements were repeated using a CM4 chip. The mAb was immobilized to a CM4 chip using three different strategies: amine coupling, aldehyde coupling, and capture. With amine coupling 127 to 477 RU of mAb was immobilized, and with aldehyde coupling 107 to 160 RU was immobilized. The resulting R_{max} values measured were approximately 11 to 43 RU and 15 to 30 RU, indicating average mAb activities of 8 and 15%, respectively. Interestingly, the aldehyde-coupled mAb still had a rather low activity considering that immobilization was through an oxidized carbohydrate. This result strongly implies that the CM–dextran layer was interacting electrostatically and blocking a fraction of the mAbs from interacting with Ag or, alternatively, preventing the Ag from binding some of the immobilized mAb due to electrostatic repulsion between the matrix and Ag. In the capture experiments, 1350 to 1750 RU of anti-human pAb was immobilized by amine coupling to the surface, and 60 to 120 RU of mAb was captured. The resulting R_{max} was 30 to 50 RU, yielding a mAb activity of 42% on average. All three CM4 immobilization protocols gave kinetic rate constants and affinity values that differed by less than 2.5-fold. However, although the affinities measured on the CM4 chip were approximately 3-fold tighter than those determined on the CM5 surfaces, the measured kinetic rate constants and affinity still varied 9- to 38-fold from the solution-phase binding parameters determined by Biacore solution-phase and KinExA experiments.

To determine whether the rate constants and affinity would move even closer to the more accurate values measured in the Biacore solution-phase and KinExA studies, we repeated our Biacore experiments using a C1 chip. Immobilization levels of the mAb by amine coupling to a C1 chip were between 178 and 257 RU with R_{max} values ranging from 20 to 37 RU, resulting in a 12% active mAb on average. Anti-human pAb was amine coupled to a C1 chip at 194 to 428 RU with 30 to 120 RU of mAb captured yielding an R_{max} ranging from 21 to 77 RU, indicating an average mAb activity of 62%, a value more in the range expected for a captured mAb. The binding parameters measured on a C1 chip were the closest of any of the Biacore surface measurements to the accurate values measured in solution for the Ag/mAb complex. Interestingly, even the results obtained on a C1 chip appeared to be affected slightly by the carboxyl groups on the sensor surface. The average results obtained from the C1 chip varied from the KinExA results (in the absence of soluble CM–dextran) in k_a, k_d, and K_D by 3.5-, 3.3-, and 12-fold, respectively. Normally, the expected differences between binding parameters determined by KinExA and surface-based Biacore experiments are in the range of 2- to 3-fold [1].

To determine the most accurate values for the k_a, k_d, and K_D in light of the discrepancies in biosensor results described above, we performed solution-based titration experiments using both KinExA and Biacore methodologies, with the latter traditionally referred to as "Biacore solution competition experiments" [1,8,15,23,24]. A Biacore solution-phase experiment is performed identically to a KinExA titration experiment except that mass detection is used to

measure free Ag rather than quantitation of free mAb binding site by fluorescence as in KinExA. For the Biacore solution-phase experiments performed in this study, Ag was held constant and mAb was titrated into Ag. It is always optimal to hold the monovalent molecule constant in a Biacore solution-phase titration because the Biacore instrument detects mass. If Ag were titrated into a constant $[mAb]_{binding\ site}$ as in KinExA, the greater mass of a mAb bound with one Ag molecule compared with an unbound mAb would lead to an erroneous signal for free mAb binding site. This artifact is avoided by holding Ag constant and titrating in mAb because Ag in this study is monovalent and will only bind to the surface if unbound in solution. Owing to the differences in the detection limits for the two methods, KinExA is capable of measuring extremely tight interactions in the femtomolar (fM) to low picomolar (pM) range, whereas double-digit pM K_D values can be challenging to measure with Biacore solution-phase experiments, hence the long injection times required to produce an acceptable signal-to-noise level for Ag binding in this work. A convenient time- and reagent-saving feature of the Biacore solution-phase titrations performed here is that no calibration curve for free Ag was required because the KinExA software package was used for fitting. This could be done because the Biacore signal is proportional to $[Ag]_{free}$ and could be plotted and fit as a function of total $[mAb]_{binding\ site}$ similarly to how the fluorescence signal is proportional to $[mAb]_{free\ binding\ site}$ in KinExA [1,8,15,24].

Even though the CM–dextran matrix must have influenced the interaction of Ag binding to immobilized mAb in the Biacore solution-phase experiments as in the Biacore surface-based kinetic measurements, this fact does not affect the final K_D calculated because complexation and equilibration take place in solution. The only purpose of the Biacore surface in this type of experiment is to quantitate free Ag. Because the equilibrated solution is constantly flowed across the sensor surface, reequilibration of a given equilibrated sample does not take place because the equilibrated solution is continually replenished. In other words, the binding reaction is kinetically excluded from reequilibrating over the flow cell similar to sample passing through the bead pack in KinExA. A flow cell in a Biacore 2000 instrument is approximately 60 nl; hence, even at a flow rate of 5 μl/min, an equilibrated sample is exposed to the surface only 0.7 s, similar to the less than 0.5 s a sample takes to flow through the bead pack in KinExA. In addition, the term "Biacore solution competition experiment" is incorrect because at no time is there ever truly a "competition" between solution and surface binding [23]. In fact, kinetic exclusion means that the equilibrium of the Ag/mAb complex in solution remains unperturbed, which allows the Biacore surface to truly quantitate the free Ag in each equilibrated solution. The KinExA and Biacore solution-phase K_D values determined agreed reasonably well. On average, they were approximately 4-fold different, where KinExA gave a K_D of 22.1 (4.7) pM, whereas Biacore gave a solution-phase K_D of 91.9 (32.4) pM.

In addition, KinExA experiments were performed to measure the K_D and k_a in the presence of soluble CM–dextran at 10 mg/ml (11 mM in carboxyl groups). Soluble CM–dextran was added to simulate, as closely as the solubility of CM–dextran would allow, the environment that the mAb might be experiencing on the sensor surface. A K_D of 60.1 (27.3) pM was measured for Ag binding to the mAb in the presence of soluble CM–dextran, which was 3-fold weaker than that observed in the absence of CM–dextran. The K_D (60.1 pM) measured with KinExA in the presence of CM–dextran was approximately 4-fold tighter than the K_D (259 pM—average of the K_D values for the covalent and capture surfaces) measured on a C1 chip. More interesting, the K_D values measured on CM5 and CM4 chips are approximately 40- and 14-fold weaker than the K_D determined with KinExA in the presence of 11-mM carboxyl groups. As discussed above and given in the Supplementary material, the carboxyl group concentration is approximately 62 to 186 mM in a CM5 dextran layer and approximately 19 to 56 mM in a CM4 dextran layer. Therefore, the molar ratios of the carboxyl group concentration in the CM5 and CM4 experiments and the corresponding KinExA titrations (11 mM) are approximately 17- and 5-fold, respectively. These ratios of carboxyl group concentration are only 2- to 3-fold different from the experimental ratios of the K_D values measured on CM5 and CM4 chips to the K_D values measured with the KinExA titrations in the presence of soluble CM–dextran. It is important to remember that the carboxyl group concentrations calculated in the Supplementary material are estimates and have been calculated using values that have been taken from the literature when the Biacore CM–dextran hydrogel was first introduced in 1990 [25]. The accuracies of the estimates of the carboxyl group concentrations outlined in the Supplementary material are unknown because the CM–dextran matrices on currently available biosensor chips may differ from those chips originally offered more than 20 years ago. For this reason, we consider the 2- to 3-fold differences observed between the ratios of the carboxyl group concentrations and the measured K_D values between Biacore and KinExA experiments to be within the error range for the approximate carboxyl group concentrations calculated in the Supplementary material. Therefore, the K_D values determined on the CM5 and CM4 sensor chips correlate reasonably with the K_D measured by KinExA in the presence of soluble CM–dextran.

The Biacore kinetic results in Table 1 show that the K_D differences measured on the three unique biosensor chips are inversely correlated to the respective k_a determinations, with only minimal alterations of the corresponding k_d values. As the amount of CM–dextran decreased with each unique type of Biacore chip, the k_a values increased an average of 10-fold and became more similar to the k_a values measured by KinExA. This result strongly indicated that for this Ag/mAb system, the k_a appeared to be greatly influenced by the CM–dextran layer. The mean k_d measured on all three Biacore chips varied with a relative standard deviation of only 21%, indicating that the negatively charged CM–dextran matrix did not have a large effect on the k_d. This is also consistent with the k_d measured by KinExA in that the k_d determined in the absence and presence of soluble dextran varied only 1.4- to 2.8-fold from that determined by Biacore. This is within the expected range of error between the two methods [1].

Fong and coworkers [27] previously reported an effect caused by the CM–dextran layer on the binding kinetics measured for a protein/protein interaction. They used sensor chips containing a CM–dextran layer and a monolayer lacking the dextran layer (a homemade chip, not a C1 chip). The authors attributed the observed differences to steric hindrance and to mass transport artifacts, both resulting from the CM–dextran matrix impeding analyte diffusion through the dextran layer. Simple visual inspection of our sensorgram data indicates that mass transport was not a factor in the kinetic results we observed from our Biacore experiments. The study conducted by Fong and coworkers made no mention of possible electrostatic effects of the CM–dextran layer with the immobilized protein, nor did they perform any solution-based measurements to establish accurate solution-phase binding parameters for their protein/protein complex. If additional experiments had been performed, they may have been useful in drawing conclusions on what additional effects the hydrogel may have had on the binding kinetics and affinity of their protein/protein interaction system.

In conclusion, we have demonstrated that it is possible to observe major differences between the binding parameters measured by Biacore and KinExA even when using optimal experimental design and analysis. The negatively charged carboxyl groups in the dextran matrix appear to be responsible for the discordance between the Biacore surface-based and KinExA

solution-phase binding measurements observed in this work. Two hypotheses exist for how the CM–dextran matrix affects the binding of this particular Ag to the mAb drug. First, the high pI of the mAb results in an electrostatic interaction between the highly positively charged mAb and the CM–dextran matrix that sterically hinders the formation of the Ag/mAb complex. Second, an electrostatic repulsion between the negatively charged CM–dextran layer and the negatively charged Ag impedes the complexation of the mAb with Ag. Indeed, it is possible that both hypotheses contribute to completely explain the CM–dextran effect.

Although many of the Biacore RU–RU_{max} relationships that we observed indicate less than optimal binding activity of mAb when covalently coupled or captured on flow cells of increasing negative charge, our current findings cannot discriminate between these two alternative hypotheses. Additional studies would be required to understand this phenomenon more clearly. One approach would be to engineer mAb variants with pI values near pH 7.0 while maintaining similar KinExA binding properties to those of the parental mAb. Once these variants were created and qualified, assessment of their kinetic binding properties by Biacore using CM5, CM4, and C1 surfaces would reveal which hypothesis is correct.

As a consequence of this extensive study, we propose three diagnostic tests that can be used to determine whether Biacore surface measurements are inaccurate. First, Biacore kinetic measurements can be made on CM5, CM4, and C1 chips to spot any troubling trends in the binding data. Large (3- to 5-fold) variations in the binding parameters when using different chips should be cause for concern. Second, a comparison between Biacore solution-phase and surface-based measurements can be performed to look for any large differences that would implicate surface kinetic artifacts. Third, KinExA solution-based measurements could be compared with Biacore surface-based measurements with large variations suggesting that a problem may exist with the surface-based measurements. These three diagnostic tests will alert researchers that their Biacore surface-based measurements could be generating artifactual data and, if so, alternative biophysical methods should be used to determine accurate binding parameters for their given protein/protein interaction.

Acknowledgments

We thank Samadhi Vitharana, Kamal Kannan, and Tian Cui for helpful discussions concerning the pI of the Ag and mAb. The Takeda San Francisco Process Sciences Department is also gratefully acknowledged for its contributions to the preparation and purification of the Ag and mAb for these studies.

Appendix A. Supplementary material

Supplementary material associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ab.2012.06.024.

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