Chapter 22

Measuring Protein–Protein Interactions Using Biacore

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Abstract

The use of optical biosensors for studying macromolecular interactions is gaining increasing popularity. In one study, 1,179 papers that involved the application of biosensor data were identified for the year 2007 alone (Rich and Myszka, J Mol Recognit 21:355–400, 2008), the sheer volume and variety of which present a daunting task for the burgeoning biosensor user to accumulate and decipher. This chapter is designed to provide the reader with the tools necessary to prepare, design, and efficiently execute a kinetic experiment on Biacore. It is written to guide the Biacore user through basic theory, system maintenance, and assay set-up while also offering some practical tips that we find useful for Biacore-based studies. Many kinetic-based screening assays require rigorous sample preparation and purification prior to analysis. To highlight these procedures, this protocol describes the kinetic characterisation of single chain Fv (scFv) antibody fragments from crude bacterial lysates using an antibody affinity capture approach. Even though we specifically describe the capture of HA-tagged scFv antibody fragments to an anti-HA tag monoclonal antibody-immobilised surface prior to kinetic analysis, the same methodologies are universally applicable and can be used for practically any affinity pair and most Biacore systems.

Key words: Antibody, Surface plasmon resonance, Biacore, Kinetics, Biosensor analysis, Protein–protein interactions.

1. Introduction

The past decade has seen radical advances in both protein discovery and antibody production methodology, creating an unprecedented need for rapid methods of studying protein–protein interactions on a molecular scale. During this period, surface plasmon resonance (SPR) biosensors, such as Biacore systems (2), have co-evolved as standard tools for protein characterisation and binding interaction studies. The potential of this maturing biosensor market is evident from the expanding number of companies providing SPR-based sensors (3), from the quantity and variety of published material using commercial biosensors

Dermot Walls and Sinéad T. Loughran (eds.), *Protein Chromatography: Methods and Protocols*, Methods in Molecular Biology, vol. 681, DOI 10.1007/978-1-60761-913-0_22,

(reviewed annually by Rich and Myszka (1, 3–11)) and as a result of the quality of Biacore data generated from recent comparative studies with methods such as KinExA (12) and solution-based calorimetry measurements (13). While Biacore-based biosensors have been used for the analysis of mammalian (14–16) and bacterial cells (17–20), viruses (21), proteins (12, 22–26), toxins (27, 28), and haptens (13, 29–33), antibody characterisation is by far the most common application (1, 3, 5–11).

1.1. Theory

Surface plasmon resonance is an optical phenomenon which occurs as a result of total internal reflection of light at a metal film–liquid interface. Under conditions of total internal reflection, incident light excites plasmons in the gold film; the angle at which this occurs (known as the SPR angle) is very sensitive to refractive index changes of the solution. Biacore biosensors measure the shift in SPR angle due to mass changes at the surface of the chip and, therefore, enable the detection and measurement of protein–protein interactions in real-time, without the use of labels. The most basic description of a biomolecular interaction between a soluble monovalent analyte (A) and immobilised monovalent ligand (B) can be interpreted as

$$[\mathrm{A}]+[\mathrm{B}]\leftrightarrows[\mathrm{AB}]$$

where one molecule [A] (usually of known concentration and injected over the surface) reversibly binds to another [B] (usually immobilised on the sensor surface) (34). The strength of the interaction can be described by

$$\frac{[\mathrm{A}]\times[\mathrm{B}]}{[\mathrm{AB}]}=K_{\mathrm{D}} \tag{1}$$

The rate of formation of complex [AB] is described by the differential equation

$$\frac{\mathrm{d}[\mathrm{AB}]}{\mathrm{d}t}=k_{\mathrm{a}}\times[\mathrm{A}][\mathrm{B}]-k_{\mathrm{d}}\times[\mathrm{AB}] \tag{2}$$

where the ratio of the association rate constant k_{a} ($\mathrm{M^{-1}s^{-1}}$) over the ratio of the dissociation rate constant k_{d} ($\mathrm{s^{-1}}$) describes the affinity constant *KA*. At equilibrium, the change in the concentration of the complex [AB] is zero, and therefore, the equilibrium dissociation constant (or affinity) K_{D} can be derived from Eq. 2 such that

$$0=k_{\mathrm{a}}\times[\mathrm{A}][\mathrm{B}]-k_{\mathrm{d}}\times[\mathrm{AB}]$$

$$k_{\mathrm{d}}\times[\mathrm{AB}]=k_{\mathrm{a}}\times[\mathrm{A}][\mathrm{B}]$$

$$\frac{k_{\mathrm{d}}}{k_{\mathrm{a}}}=\frac{[\mathrm{A}][\mathrm{B}]}{[\mathrm{AB}]}=(\text{from Eq.1 above})K_{\mathrm{D}}$$

2. Materials

The materials listed in this section may be obtained from Biacore, GE Healthcare, Uppsala, Sweden unless otherwise stated.

2.1. Instrument Cleaning and Surface Preparation

1. Biacore CM5 and maintenance sensor chips.
2. BIAdesorb solution 1 consisting of 0.5% (w/v) SDS.
3. BIAdesorb solution 2 consisting of 50 mM glycine–NaOH, pH 9.5.
4. HBS running buffer (10 mM HEPES, 150 mM NaCl, 3 mM EDTA, and 0.05% (v/v) surfactant P20).
5. Ultrapure H_2O.
6. 100 mM HCl and 50 mM NaOH.

2.2. Ligand Preconcentration

1. HBS running buffer.
2. 10 mM Sodium acetate pH 4–5 with 10% (v/v) acetic acid.
3. Anti-HA tag monoclonal antibody (Affinity BioReagents).
4. 20 mM NaOH.

2.3. Immobilisation

1. HBS running buffer.
2. 0.4 M EDC (*N*-ethyl-*N*′-(dimethylaminopropyl)carbodiimide).
3. 0.1 M NHS (*N*-hydroxysuccinimide).
4. Anti-HA tag monoclonal antibody diluted in sodium acetate buffer at the appropriate pH.
5. 1 M Ethanolamine–HCl, pH 8.5.
6. 20 mM NaOH.
7. *Optional:* Control protein for immobilisation onto the reference flow cell.

2.4. Surface Regeneration

1. HBS running buffer.
2. Full range of regeneration buffers.
3. Anti-HA tag monoclonal antibody.

2.5. Kinetic Studies and Data Analysis

1. HBS running buffer.
2. Bacterial lysate containing expressed scFv antibody fragment.
3. Purified monomeric analyte of choice.
4. 120 mg/mL CM Dextran in HBS running buffer.
5. 120 mg/mL bovine serum albumin (BSA) in HBS running buffer.
6. Optimised regeneration buffer.

3. Methods

Although a panoply of sensorgrams, assay formats, and detailed protocols can be found described in the literature from a variety of instruments (35–39), the analysis of antibody/antigen complexes using Biacore technology usually requires the immobilisation of one interactant (in relatively pure form) on the sensor chip surface followed by the injection of the second interactant, the concentration of which must be known for the calculation of the association constant, k_a. Affinity-based immobilisation (affinity capture) (23–25) is an alternative format that facilitates surface regeneration and the directed orientation of the antibody (40). Essentially, the affinity-based immobilisation protocol we describe involves the non-reversible immobilisation (amine coupling) of a high-affinity ligand (anti-HA tag monoclonal antibody) to reversibly anchor recombinant HA-tagged scFv antibody fragments to the surface prior to analyte injection. Replacement (regeneration) of the scFv antibody fragment after each binding cycle avoids losses in antibody binding activity, assuming scFv antibody capture is reproducible (see Subheading 3.4 on surface regeneration).

Regardless of experimental design, the quality of one's biosensor data is always directly dependent on the quality of the reagents used (41). While capture assays can be used to rank antibodies from crude matrices (25), the analyte should be well characterised (exact concentration known), of high purity (>95%), of good integrity (functional with no aggregates), and for 1:1 interaction studies be monovalent. The Biacore user should be fastidious in instrument preparation (discussed in Subheading 3.1), spend time choosing the right assay set-up for their interaction pair (see Note 1), try to eliminate artefacts such as non-specific binding (see Note 2), evaluate the impact of wash (see Note 3) and regeneration steps (see Note 4), and always include sample replicates (see Note 5).

3.1. Instrument Cleaning and Surface Preparation

The method described in this chapter was performed on a Biacore 3000 instrument, but irrespective of the instrument used, proper and regular system maintenance is essential when aiming to generate high-quality data. In addition to the recommended "desorb" and "sanitize" maintenance protocols listed in the Biacore 3000 handbook, we recommend performing a "super-desorb" (13, 26, 42) followed by preconditioning of a fresh CM5 sensor chip.

1. Undock the instrument sensor chip and dock a maintenance chip (see Note 6).
2. Prime the instrument five times with 0.5% (w/v) SDS (BIAdesorb solution 1).

3. Replace the 0.5% (w/v) SDS solution with ultrapure water and prime the system once.
4. Prime the instrument five times with 50 mM glycine–NaOH, pH 9.5 (BIAdesorb solution 2).
5. Replace the 50 mM glycine–NaOH, pH 9.5 solution with ultrapure water and prime the system another five times.
6. Undock the instrument maintenance chip and dock a fresh CM5 sensor chip and prime three times with running buffer.
7. Set the instrument flow rate to 100 µL/min and precondition the chip with two consecutive injections each of 100 mM HCl, 50 mM NaOH, and 0.5% (w/v) SDS for 10 s per injection.

3.2. Ligand Preconcentration

Low ionic strength buffers allow the electrostatic adsorption of positively charged proteins (proteins in low ionic buffer at a pH below the proteins' isolectric point [pI]) to the negatively charged dextran surface, maximising "*preconcentration*" of protein to the sensor surface for subsequent immobilisation and thus increasing the amount of ligand at the surface that can be efficiently covalently coupled on suitably activated chip surfaces. Electrostatic binding of protonated amine groups on the biological component to negatively charged carboxyl groups on the chip surface is facilitated by adjusting the pH below the pI of the protein. Therefore, protein solutions should be prepared in low ionic strength buffers such as 10 mM sodium acetate at a range of different pHs and these solutions passed over an underivatised chip surface, with the degree of electrostatic binding monitored. The highest pH (see Note 7) at which preconcentration of protein onto the underivatised surface is observed to be sufficient should be chosen as the pH for immobilisation. Depending on user preference, the methods described below can be performed using the manual operation function, the application wizards, or the method control function.

1. Prepare 25 µg/mL anti-HA tag monoclonal antibody (see Note 8) in 10 mM sodium acetate buffer at pH increments from pH 4 to 5.
2. Inject each sample over flow cell 2 of a preconditioned CM5 sensor chip (see Subheading 3.1) for 30 s at a flow rate of 10 µL/min (see Note 9).
3. *Optional:* If the response does not return to baseline after ligand preconcentration (some ligands can have a tendency to non-specifically stick to the dextran surface), inject 20 mM NaOH for 15 s after each preconcentration.
4. Evaluate the magnitude and slope of the preconcentration response (see Fig. 1) and determine if the desired ligand density can be achieved. Use the mildest pH conditions that will enable the achievement of the desired immobilisation level.

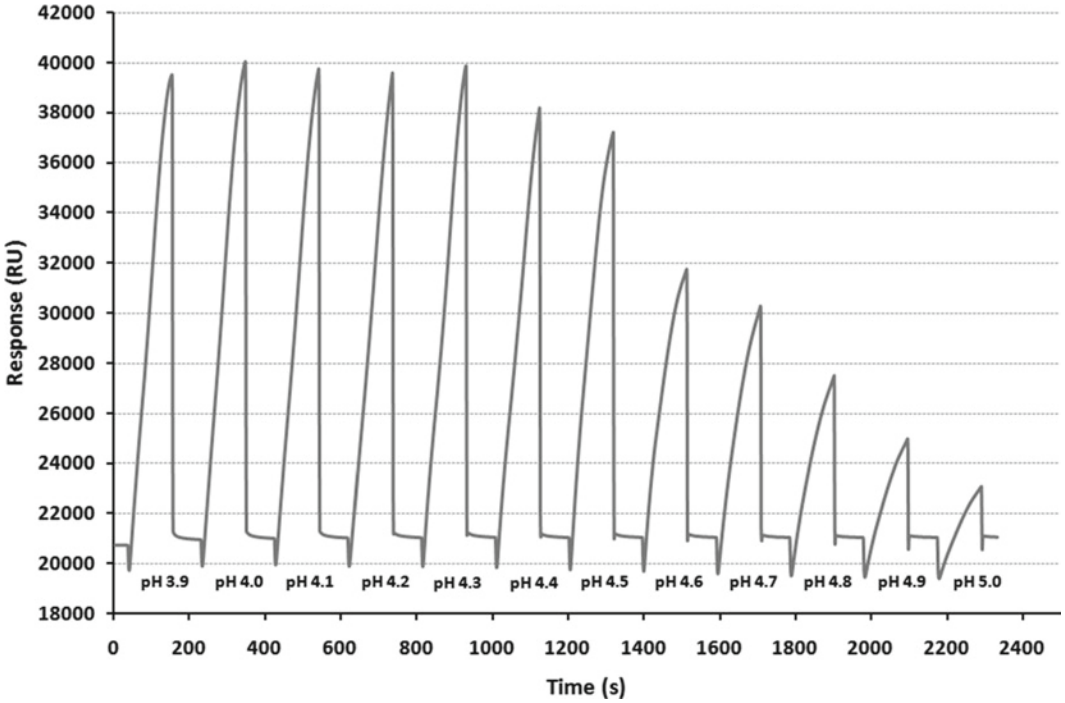

Fig. 1. Typical preconcentration profile of a monoclonal capture antibody electrostatically interacting with the CM-dextran sensor chip surface. Solutions of 50 μg/mL of antibody in 10 mM sodium acetate buffer at various pH increments (indicated on the sensorgram shown here) were passed over an untreated CM-dextran surface at 10 μL/min for a period of 2 min. The low ionic strength of the acetate buffer favours the electrostatic attraction between the negatively charged dextran layer and the positively charged protein (i.e. below its isoelectric point). The degree of preconcentration was measured from the response prior to the end of each sample injection with the ionic strength of the HBS running buffer (containing 150 mM NaCl) sufficient for the removal of electrostatically attracted antibody from the surface. The pH used for the high density immobilisation (>10,000 RU) of the monoclonal antibody onto the CM-dextran chip surface was pH 4.5 (see Note 7).

3.3. Immobilisation

Immobilisation of ligand to a CM5 sensor chip surface involves activation of the surface, followed by ligand preconcentration/immobilisation and finally surface deactivation. Even though there are a wide variety of immobilisation chemistries available for the coupling of ligands to the CM dextran surface, depending on the functional groups available for coupling, the most commonly employed strategy is the use of EDC (*N*-ethyl-*N*′-(dimethylaminopropyl) carbodiimide) and NHS (*N*-hydroxysuccinimide) chemistry. Using the carbodiimide EDC, the CM dextran carboxyl groups can be transformed into active ester functional groups in the presence of NHS. The surface NHS esters can then react with suitable amino groups in the protein and deactivated or "capped" by the injection of 1 M ethanolamine–HCl (pH 8.5), which also serves to remove non-covalently bound protein by reducing the electrostatic attraction between the protein and the CM-dextran (carboxymethyl dextran sodium salt).

1. Prepare 100 µL of 0.4 M EDC, 100 µL of 0.1 M NHS, 200 µL of 25 µg/mL anti-HA tag monoclonal antibody (capture antibody), and 100 µL of 1 M ethanolamine–HCl.
2. Mix equal volumes of 0.4 M EDC and 0.1 M NHS and inject 70 µL over flow cell 2 (same flow cell as preconcentration) at 10 µL/min flow rate (see Note 10).
3. Inject 150 µL of capture antibody over the activated surface at 10 µL/min (see Note 11).
4. Inject 70 µL of 1 M ethanolamine–HCl at a flow rate of 10 µL/min to block residual activated carboxyl moities on the surface.
5. Post-condition the surface with five 15 s injections of 20 mM NaOH.
6. *Optional:* Prepare a reference surface on flow cell 1 by repeating steps 1–5 above replacing the capture antibody in step 3 with 25 µg/mL of suitable control protein of choice (see Note 12).

3.4. Surface Regeneration

The efficient and reproducible regeneration of antibody-binding surfaces is of major importance for reusable sensor formats such as required for direct "real-time" biosensing technologies and is often difficult to achieve (40). For accurate kinetic analysis, surface regeneration while maintaining ligand integrity is usually of paramount importance (see Note 13). Surface regeneration studies usually involve the identification of a suitable regeneration buffer (regeneration scouting) and the performance evaluation of the surface (surface performance test). The aim of the regeneration studies is to identify a suitable buffer to remove all the bound analyte without compromising functionality of the surface ligand. In order not to destroy the ligand surface, it is always recommended to start buffer scouting with mild buffers working up to more harsh buffers if regeneration is not readily achieved. Once a suitable buffer (or possibly a number of buffers) is identified, its effect on ligand functionality over time is evaluated by repeated binding studies to determine its suitability for kinetic studies (i.e. does it maintain a fully active surface over the study life time?). For multiple analyte screening, capture kinetic experiments simplify regeneration studies as conditions for only one binding interaction (the capture ligand binding interaction) are required compared to direct binding studies where the vast range of analyte affinities may require different buffer conditions to achieve regeneration.

3.4.1. Regeneration Scouting

1. Prepare a panel of regeneration buffers to be tested (see Note 14).
2. Inject 60 µL of a high concentration of HA-tagged scFv antibody (see Note 15) over the anti-HA tag monoclonal

antibody immobilised surface and reference surface at 30 µL/min. Monitor both the baseline and binding levels.

3. Starting from the mildest buffer, inject 15 µL of the regeneration buffer at 30 µL/min.
4. Repeat steps 2 and 3 five times and then change to the next mildest buffer.
5. Follow the trend in scFv-capture response (relative response measured from the baseline before injection of the scFv) and the absolute baseline level (see Fig. 2).

3.4.2. Surface Performance Test

1. Prepare 1 mL regeneration buffer identified in Subheading 3.4.1 and 2 mL scFv antibody lysate.
2. Inject 30 µL scFv antibody over flow cells 1 and 2 (reference and capture surfaces) at 30 µL/min. Monitor both the baseline and binding levels.
3. Inject 15 µL regeneration buffer over flow cells 1 and 2 at 30 µL/min.
4. Repeat the binding/regeneration cycle for 30–40 cycles or as required for the number of cycles to be used in the final assay.

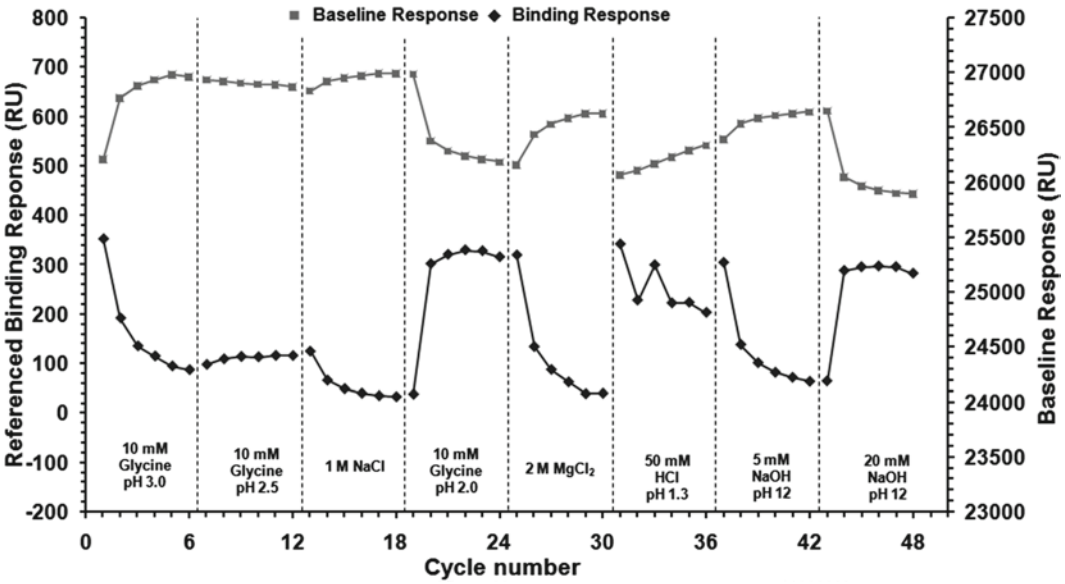

Fig. 2. Anti-HA tag antibody surface performance study using repeated scFv lysate binding cycles regenerated with mild to harsh buffer conditions (indicated on the graph). Six binding cycles for each buffer were performed and the baseline (*squares*) and binding response (*diamonds*) levels recorded. A decrease in binding response with an increase in baseline response was seen with 10 mM glycine, pH 3.0, 1 M NaCl, 2 M $MgCl_2$, 50 mM HCl, pH 1.3, and 5 mM NaOH, pH 12, indicating these buffers were too mild. While both the baseline and binding response levels for 10 mM glycine, pH 2.5, were stable, only 10 mM glycine, pH 2.0, and 20 mM NaOH, pH 12, restored both the baseline and binding responses back to original levels in cycle 1. The latter two buffers were subsequently evaluated as described in Subheading 3.4.2 and 20 mM NaOH chosen as the regeneration buffer for kinetic analysis.

5. Evaluate the ligand stability and baseline level over time. If the baseline increases (with a subsequent decrease in binding level), a harsher regeneration solution may be needed. If the binding capacity decreases significantly while the baseline remains constant or even decreases, the ligand stability is being compromised resulting in a lower analyte binding capacity and a different regeneration solution should be used. Regeneration studies, albeit tedious, are essential for the optimum assay performance and, therefore, time invested in identifying the correct conditions is time well spent.

3.5. Kinetic Studies and Data Analysis

The design of a kinetic experiment must include sufficient experimental data for the reliable calculation of accurate rate constants. Experimental parameters such as (a) temperature, (b) immobilisation level, (c) injection time and flow rate, (d) dissociation time and (e) analyte concentration range should be evaluated prior to running the kinetic experiment (35). Experimental design should also include blank injections for elimination of systematic artefacts, repeat analyte injections, and start up cycles to condition the flow system and sensor surface (35, 41).

1. Prepare doubling dilutions of analyte from 100 to 1.56 nM (see Note 16) in running buffer and include a buffer sample for double referencing to remove instrument artefacts.
2. Dilute bacterial lysate containing expressed scFv antibody 1/10 in running buffer containing 12 mg/mL BSA and 12 mg/mL CM dextran.
3. Perform three to five "*start up*" cycles to confirm that the surface performance is reproducible and to condition the flow system and surface.
4. Inject a sufficient concentration of bacterial lysate, containing expressed scFv, to give an analyte R_{max} of approximately 50 RU (response units) (see Note 17). For bacterial lysates expressing avian scFv antibodies, we use a 2–3 min injection at 20 µL/min.
5. Inject 90 µL of 100 nM analyte at 30 µL/min and allow dissociation in buffer for 10 min.
6. Inject 15 µL of optimised regeneration solution at 30 µL/min.
7. Repeat steps 4–6 above three to five times and evaluate the binding responses which should be reproducible.
8. Perform the kinetic analysis on the range of analyte concentrations listed in step 1, running at least one analyte concentration in duplicate or triplicate and running a buffer injection for double referencing.

9. Inject 60 μL of bacterial cell lysate containing scFv (prepared in step 2) at 20 μL/min over flow cells 1 and 2 (over the reference and active surface).
10. For each analysis cycle, inject 90 μL of analyte (inject analyte concentrations randomly including replicates and buffer injections) at 30 μL/min and allow dissociation in buffer for 15–20 min.
11. Inject 15 μL of optimised regeneration solution at 30 μL/min.
12. *Optional:* After regeneration, perform an IFC (integrated micro fluidics cartridge) wash procedure followed by short buffer injection to clean the injection needle, tubing, and flow path (see Note 3).
13. Repeat steps 8–12 above until all analyte concentrations have been analysed.
14. Open the above kinetic analysis experimental result file in BIAevaluation and prepare the raw data for analysis.
15. Overlay the reference-subtracted data and adjust the baseline prior to analyte injection to zero and align the overlaid sensorgrams with time on the *x*-axis.
16. Remove the regeneration data, capture data, and any other unnecessary data from the sensorgram.
17. Subtract the zero analyte response from the analyte responses ("*double referencing*") to remove systematic deviations.
18. Select the interaction model that best suits your data and start the fitting procedure. The curve fitting procedure is an iterative mathematical process whereby the evaluation software, using default starting values for the parameters to be determined, calculates theoretical binding curves and compares them with experimental data by least square fitting of residuals (see Fig. 3).

4. Notes

1. When designing a binding assay on Biacore, one of the interactants is generally captured or immobilised onto the surface (the ligand) and the other interactant passed over the surface (the analyte). Choosing which interactant from an affinity pair to immobilise will depend on its stability (effect of immobilisation and regeneration on its function), its non-specific binding (NSB) tendency (proteins with a high pI tend to show NSB to the dextran surface and are better used as the ligand), its valency (if possible always use a monovalent

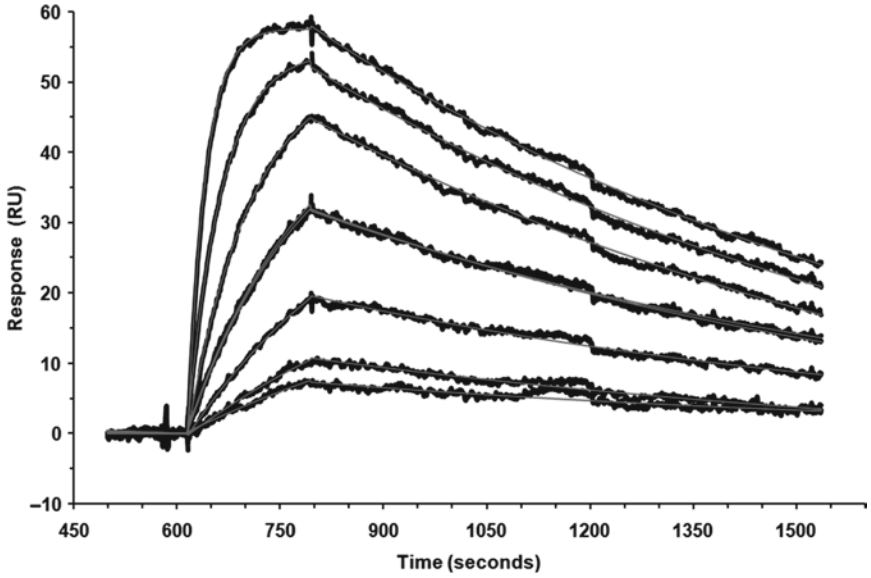

Fig. 3. Example of fitted kinetic data from the analysis of a captured HA tagged avian scFv antibody fragment binding to a human disease protein biomarker (unpublished work using the method described in Subheading 3.5). The fitted curves (fitted with a 1:1 with drifting baseline model) are shown as *solid thin grey lines* overlaid on the raw analyte responses (*thick black lines*) at 100, 50, 25, 12.5, 6.25, 3.125, and 1.56 nM (the 12.5 nM analyte concentration was analysed in duplicate). The values of the calculated kinetic parameters were 3.72×10^5 $M^{-1}s^{-1}$ (k_a), 1.07×10^{-3} s^{-1} (k_d), and 2.88×10^{-9} M (K_D).

molecule as the analyte), and its size (larger molecules are preferred as the analyte even though, with good experimental design, the latest Biacore instruments are capable of measuring binding responses from analytes as small as 100 Da [see http://www.biacore.com]).

2. Prior to assay set-up, evaluation of NSB is required as many biological materials can often exhibit a tendency to non-specifically bind to the sensor surface. This can be done by simply injecting each reagent separately over a blank surface at the highest concentration to be used in the assay. If NSB is high, consider changing the experimental buffers (e.g. adding 12 mg/mL CM dextran and 12 mg/mL BSA in the sample buffer can help reduce NSB) or changing the sensor chip chemistry (e.g. use another sensor chip type or consider deactivating with ethylenediamine instead of ethanolamine after amine coupling).
3. Kinetic analysis, especially direct binding studies (i.e. not following a capture step) can be complicated by small amounts of regeneration buffer carried over between binding cycles which can contaminate the sample plug and affect the next binding reaction (41). Performing a wash step and buffer injection between cycles can help eliminate this.

4. Maintaining ligand function while fully removing bound analyte is essential for reproducible kinetic experiments, and considerable effort spent scouting the right conditions will pay dividends in the quality of the kinetic data obtained.
5. Replicate analyses (duplicate or triplicate) and analyte randomisation are necessary to show the repeatability of the assay, the regeneration step, and ligand activity over time.
6. During the desorb procedure, any proteins on the sensor chip surface are destroyed. In addition, a maintenance chip (contains glass only) should be docked to avoid the possibility of stripping small amounts of gold off the sensor chip which could stick to the instrument IFC.
7. The immobilisation pH to use should be the highest pH that gives suitable immobilisation levels. The buffer pH to be used will depend on the immobilisation level required (low pH buffer may be needed to provide adequate preconcentration of sample for high capacity surfaces used for concentration analysis studies, while lower levels and, thus, milder pH can be used for kinetic surfaces), the sample stability (some proteins are not stable at low pH), and sample concentration (some samples may be in limited supply and maximum preconcentration conditions will be needed).
8. To avoid possible NSB and assay interference, the ligand immobilised should be at least 95% pure if possible. Many commercial antibodies come with additional stabilisers which can contaminate and reduce immobilisation efficiency. If these are present, the antibody should be purified by affinity chromatography (see Chapter 3).
9. The protein concentration to use for preconcentration studies will depend on ligand availability and desired immobilisation level. Generally, proteins are diluted to 1–50 μg/mL in appropriate buffer.
10. Approximately 40% of the carboxyl groups on a CM5 chip surface are activated after a 7 min activation injection. If high-density surfaces are required, this injection time can be increased.
11. The injection times and immobilisation level needed for capture experiments will vary with assay requirements and ligand properties. If the concentration of the scFv antibody to be studied is known *a priori*, the desired anti-HA capture antibody immobilisation levels could be theoretically calculated (see Note 16). In this experiment, we aimed to characterise hundreds of antibody clones differing over 1,000-fold in antibody expression levels and, therefore, a high capacity surface (~10,000 RU) was chosen.

12. On Biacore, the reference surface may be (a) an unmodified surface, (b) an activated-deactivated surface, or (c) a surface immobilised with a non-interacting "dummy" ligand. A prerequisite for choosing which reference surface to use is the determination of the level of NSB to the surface. This should be performed by injecting the analyte at the highest concentration over the reference surface while evaluating the binding levels, if any. If non-specific binding is not an issue, try treating the reference surface with the same immobilisation levels and conditions used for the active test surface as this creates a similar environment between the flow cells.
13. Of increasing popularity is the sequential analysis of binding interactions with reduced regeneration cycles ("*single cycle kinetics*") for kinetics (43) and concentration analysis (44) or accurately choosing a small number of analyte concentrations that are confidently measured (34). These approaches can be very useful where regeneration scouting is difficult due to ligand instability.
14. We usually prepare and filter a range of solutions such as 10 mM glycine buffers, pH 1.5–3.0, 10–100 mM phosphoric acid, HCl, and NaOH and high ionic strength buffers such as 1 M NaCl and 2 M $MgCl_2$.
15. Inject a concentration of HA-tagged scFv antibody equal to or greater than the highest concentration used in the study. This could be a stock lysate of a high expression clone.
16. As a general rule, multiple analyte concentrations ranging from 0.1 to 10 times K_D should be used for kinetic studies with at least one (or preferably more) concentration performed in duplicate. From our experience, the affinity of antibody clones stringently selected (with no *in vitro* affinity maturation steps incorporated) from immune antibody libraries generally range from ~50 to 0.5 nM. Therefore, choosing an analyte concentration range that can be "confidently measured" (34) for *all* clones in a high-throughput screening run will depend on the screening criteria set by the user (i.e. if detailed kinetic analysis is needed, a large range of concentrations will have to be analysed and those concentrations that can be confidently measured used for curve fitting and statistical analysis. However, if the experimental aim is to kinetically rank a high number of clones, a lower analyte concentration range can be used and selected clones reanalysed over an analyte range more suited to its affinity).
17. Information on the optimal ligand immobilisation level relative to binding stoichiometry [binding stoichiometry = (analyte R_{max}/ligand density)×(ligand MW/analyte MW)], where MW is molecular weight, required to achieve the most appropriate maximum analyte binding response

(R_{max}) for kinetic analysis (i.e. <100 RU). Combining low R_{max} levels with high analyte sample flow rates is essential to counteract mass-transfer limitations. If the experimental stoichiometric R_{max} is less than expected, this can indicate that the ligand has lost some activity, a commonly seen artefact with random immobilisation coupling chemistries (37). When multi-valent interaction partners (e.g. bivalent IgG) are to be studied, they should preferentially be used as the surface-ligand in kinetic experiments. If the cognate analyte is truly monomeric then the interaction can be reliably studied using a 1:1 model. For the analysis of a low number of samples, the experimental scFv capture level required to achieve the desired R_{max} can be determined by the iterative injection of diluted samples over the capture surface. However, for high-throughput screening of hundreds of antibodies from crude bacterial lysates, with greater than 1,000-fold differences in expression level, this is not practical, and from our experience, a 2–3 min injection of a 1/10 dilution of scFv containing lysate injected over a high capacity anti-HA monoclonal antibody surface is sufficient for most avian scFv antibody clones.

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