Kinetic analysis of antibody binding to an expressed membrane protein on captured lipoparticles

Bettina Heidecker, Scientist, Krista Witte, Senior Director, Wesley McGinn-Straub, data courtesy of Integral Molecular

Introduction

Membrane proteins govern the majority of input and output signals of cells and represent the largest class of pharmaceutical drug targets, making the analysis of their molecular interactions critical to mapping the interactome as well as drug discovery efforts. Due to their integration into a lipid bilayer, in vitro characterization of molecular interactions of membrane proteins presents a unique challenge compared to soluble proteins. By capturing 150 nm-sized lipoparticles that contain a targeted membrane protein on the surface of a biosensor, ForteBio's Octet® platform enables the kinetic analysis (k_{on}, k_{diss} and K_D) of membrane protein-analyte interactions. Due to the large size of the lipoparticle, the observed data trace is often inverted, requiring a flip during data processing. Using a membrane protein-antibody model system, data processing and analysis of the membrane protein-analyte interaction on a lipoparticle is discussed and validated.

Lipoparticles

Lipoparticles, available from Integral Molecular (www.integralmolecular.com), offer expression of integral membrane proteins in a native lipid bilayer format for nanoscale in vitro protein analysis research (Figure 1). The proprietary retroviral-based technology incorporates a high concentration of conformationally intact membrane protein into a ~150 nm virus-like particle (VLP) composed of natural cell membranes. This cell-free solution for membrane protein analysis creates a unique biochemical tool to study GPCRs, ion channels, and other membrane proteins. Lipoparticles do not contain a genome and are thus non-infectious.

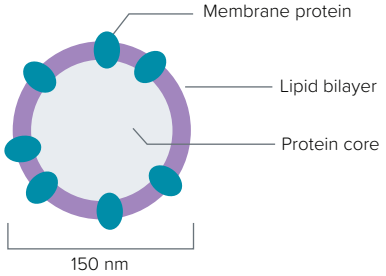


Figure 1: A membrane protein of interest expressed into a lipid bilayer lipoparticle, prepared using a proprietary technology from Integral Molecular and ready for *in vitro* interaction analysis. The dense protein core of the lipoparticle provides a biologically relevant context at the inner membrane surface.

Octet platform and BLI

ForteBio's label-free detection platform provides instruments, biosensors, reagents and assay kits for analyzing biomolecular interactions in 96- and 384-well microplate formats. A proprietary Bio-Layer Interferometry (BLI) technology enables real-time analysis of interactions on the surface of a fiber optic biosensor (Figure 2), providing information on affinity, kinetics and concentration. The efficient workflow of the platform provides easy, fast and high-quality characterization of drug candidates in drug development applications where existing methods have limitations in throughput, performance and cost.

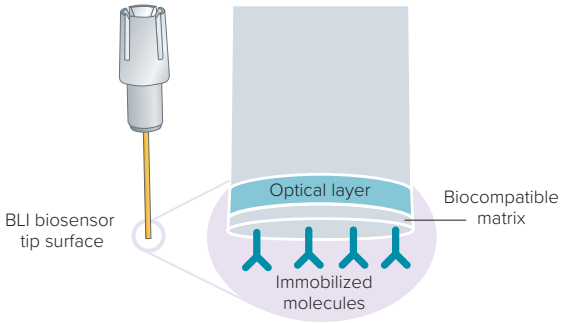


Figure 2: ForteBio's fiber optic sensors contain a biocompatible matrix layer on their bottom surface. Immobilization of molecules of interest within the biocompatible matrix enables kinetic characterization of molecular interactions and quantitation of analytes.

Workflow

Biomolecular analysis of the interaction between a lipoparticle associated membrane protein and an antibody begins with immobilization of the lipoparticle upon the biosensor surface. This example exploits the high affinity between wheat germ agglutinin and lipoparticle carbohydrates for lipoparticle immobilization. The basic experiment contains 6 steps (Figure 3): Step 1, biosensor hydration; Step 2, immobilization of biotinylated wheat germ agglutinin (WGA) on a ForteBio Streptavidin Biosensor; Step 3, capture of the VLP utilizing the immobilized WGA; Step 4, wash and establish baseline; Step 5, measure membrane protein-antibody association kinetics; and Step 6,

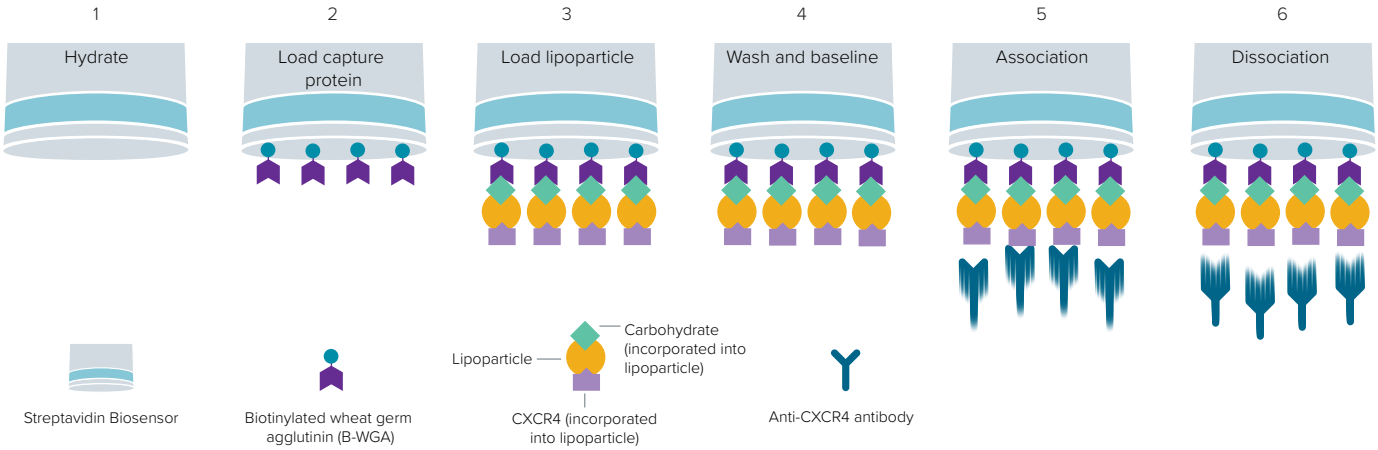


Figure 3: Example workflow for kinetic characterization of the interaction between the CXCR4 membrane protein and an anti-CXCR4 antibody. Step 1: Streptavidin Biosensor hydration, Step 2: Load biotinylated wheat germ agglutinin, Step 3: Immobilization of the CXCR4 containing lipoparticle, Step 4: Baseline in buffer, Step 5: Measure association kinetics between anti-CXCR4 antibody and lipoparticle-displayed CXCR4, Step 6: Measure dissociation kinetics between anti-CXCR4 antibody and lipoparticle-displayed CXCR4.

measure membrane protein-antibody dissociation kinetics. Alternative methods of lipoparticle capture or immobilization, such as amine coupling, are available.

Tips for optimal performance

- Octet RED96e, HTX, and RED384 instruments are recommended.
- 180 µL to 200 µL/well of reagent is required in a 96-well microplate. 100 µL/well is required for a 384-well microplate and 40 µL/well is required for a 384-tilted well plate.
- Hydrate biosensors in assay buffer for at least 10 minutes prior to starting the experiment.
- Evaluate shake speeds between 500 and 1000 rpm for optimal performance with each ligand-analyte system.
- Due to their large size, lipoparticles can generate a negative response in biolayer interferometry and, consequently, all binding events subsequent to the loading step may also be expected to generate negative responses. This negative response can be managed in the Octet Data Analysis software (Version 6.3 or greater) by signal inversion using the flip feature, followed by standard processing protocols.
- Employ one of three referencing methods:
  - Use null lipoparticles (without over-expressed target membrane protein) as a reference biosensor either as a single biosensor in a column of biosensors (reference biosensor) or as a parallel column of biosensors against the same analyte series (parallel reference subtraction).
  - Use receptor-containing lipoparticles to probe a reference well of buffer (no analyte or negative control antibody).
  - Combine the use of reference biosensor(s) and a reference well with double referencing.
- Choose controls that closely match the analytes of interest (*e.g.*, if testing murine-derived antibodies, the control should be a murine-derived antibody with an epitope not present on the protein of interest or the lipoparticles).
- If inter-step correction is to be used during data processing, the same well should be for both baseline and dissociation steps.

Required reagents

- Streptavidin (SA) biosensor Tray (ForteBio part no. 18-5019)
- Black 96-well (Greiner part no. 655209), 384-well (Greiner part no. 781209) or 384-tilted well (ForteBio part no. 18-5080) polypropylene plate
- PBS supplemented with 1 mg/mL BSA (PBS-B; BSA Fraction V, Sigma part no. A3059, 0.2 µm filtered)
- Biotinylated Wheat Germ Agglutinin (WGA) (Vector Laboratories part no. B-1025)
- 1 to 7 units lipoparticles (ligand) per biosensor
- Antibody (analyte) of interest
- Negative control analyte

Optional reagents for regeneration

- 1% Empigen (Sigma part no. 45165)
- 500 mM NaCl
- 500 mM N-acetyl-D-glucosamine (NAG) (Alfa Aesar part no. A13407)
- 10 mM HCl

Assay protocol with single reference well

SAMPLE PLATE PREPARATION

Prepare a microtiter plate (96-well, 384-well or 384-tilted well) with the required ligands, analytes and wash buffers (refer to Figure 4A for representative plate setup for single reference well subtraction).

All steps in the following example were performed at a shake rate of 600 rpm, except for the association and dissociation steps, which were performed at a shake rate of 1000 rpm. Optimal shake speeds may vary.

PREPARE AND LOAD BIOSENSOR SURFACE WITH WGA

1. Move biosensors to PBS-B (Figure 4A, column 1) and equilibrate for 5 minutes.
2. Perform a 3-minute wash step (Figure 4A, column 2).
3. Load biosensors with WGA by incubating with 50 µg/mL biotinylated WGA in PBS-B (Figure 4A, column 3) for 3 minutes.
4. Quickly rinse biosensors by running a 3-minute baseline in PBS-B (Figure 4A, column 4).

LOAD LIPOPARTICLES ON BIOSENSORS

1. Load lipoparticles for 30 minutes. Typically, the lipoparticles are diluted to a concentration of 1–10 µg/mL in PBS-B with 1–7 units of membrane protein per well (Figure 4A, column 5). A shift of 1 to 2 nm during target loading is expected. The sign of the observed nm shift may be negative due to the large size of the lipoparticles. Loading levels can be modulated by varying either the particle concentration or the loading time.
2. Place biosensors back in PBS-B (Figure 4A, column 6) and wash for approximately 45 minutes. The quality of any subsequent kinetic data is strongly dependent on baseline stability. Higher levels of lipoparticle loading may require longer stabilization times.

ASSESS ANTIBODY BINDING KINETICS

1. Using a fresh column of PBS-B (Figure 4A, column 6), record a baseline reading for 3 minutes or longer.
2. Place biosensors in antibody dilution series (Figure 4A, column 7) and measure the association for 5 minutes. A longer association phase may be needed if the association rate of the analyte is slow or if equilibrium binding is being measured.
3. Place biosensors back in PBS-B (Figure 4A, column 6). Measure dissociation for 30 minutes (the dissociation time can be adjusted as needed). Use of the same well for baseline and dissociation steps is required if inter-step correction is applied during data processing.

REGENERATION (OPTIONAL)

1. Biosensors can be regenerated back to the WGA surface (either on- or off-line).
2. Place biosensors in a solution of 1% Empigen/500 mM NaCl/500 mM NAG for 1 minute (Figure 4A, column 8).
3. Place biosensors in 10 mM HCl for 1 minute (Figure 4A, column 9).
4. Repeat Steps 1 and 2 three to five times.
5. Rinse biosensors in PBS or assay buffer to remove residual detergent or acid (Figure 4A, column 10).

Raw data

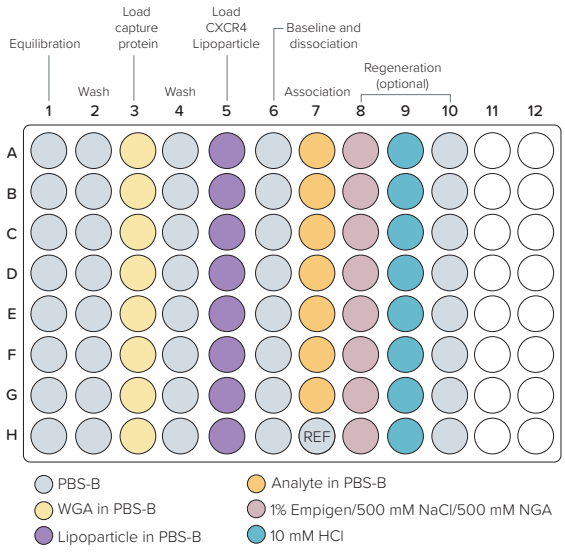
Kinetic data for the interaction of lipoparticle-displayed CXCR4 and the mouse anti-CXCR4 antibody was obtained in a 9-step assay (Figure 5). After biosensor equilibration and wash steps, loading of the biotinylated WGA produced a large positive shift of approximately 3.5 nm (Figure 5, Step 3). Excess WGA was washed away (Figure 5, Step 4) and the CXCR4 displaying lipoparticles were loaded (Figure 5, Step 5). Due to the large size of the lipoparticles, the amplitude of the signal generated by their binding to the biosensor was negative, inducing an observed amplitude shift from approximately 3.5 nm to approximately 1.75 nm. The lipoparticle-loaded biosensors were washed extensively (Figure 5, Step 6) in preparation for establishing a baseline signal (Figure 5, Step 7). Lastly, the kinetics of association (Figure 5, Step 8) and dissociation (Figure 5, Step 9) for the interaction between the anti-CXCR4 antibody and the lipoparticle-displayed CXCR4 were measured. The nm shift of the association and dissociation steps were significantly smaller than the nm shift than the loading steps, and are therefore expanded in Figure 6A and 6B for improved visualization.

Data processing

The raw data acquired for the interaction between the lipoparticle- displayed CXCR4 and the anti-CXCR4 antibody was processed and fit to a curve in order to extract values of k_{on}, k_{diss} and K_D. Processing began with reference correction to compensate for signal drift of the immobilized biosensor with the assay buffer. Using a reference biosensor, the signal generated by a biosensor with CXCR4- containing lipoparticles that probed a blank matrix (PBS-B) was subtracted from both the association and dissociation steps for the interaction between the lipoparticle-displayed CXCR4 and the anti-CXCR4 antibody. Subsequently, Y-axis alignment, inter-step correction and Savitzky-Golay filtering were applied.

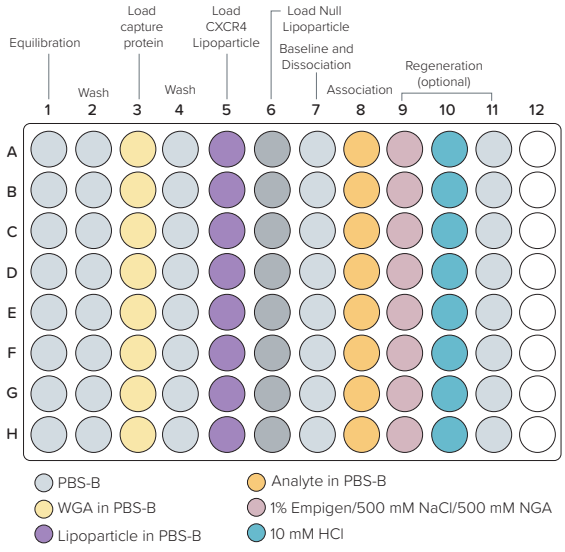
A

Sample plate setup for experiment using single reference well subtraction



B

Sample plate setup for experiment using parallel reference sensor subtraction



C

Sample plate setup for experiment using double reference subtraction

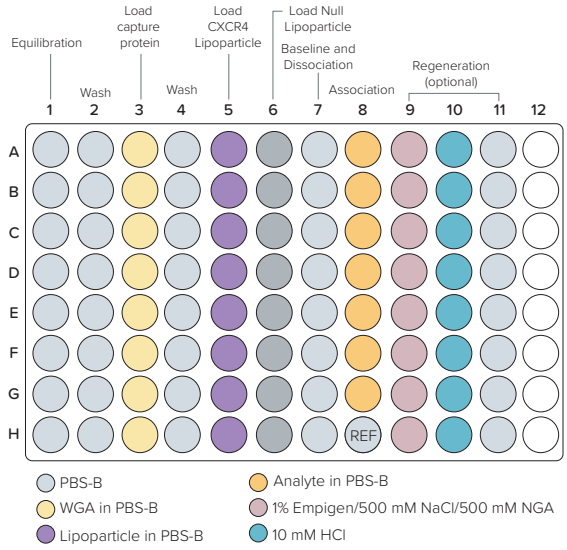


Figure 4: Sample plate setups for measuring the kinetics for the interaction between the membrane protein CXCR4 and an anti-CXCR4 antibody with different reference subtraction methods. Reagents required for regeneration are optional. The assay buffer used is PBS-B (PBS with 0.1 % BSA). If inter-step correction is to be applied during data processing, the same well should be utilized for both the baseline and dissociation steps.

Panel A represents an assay utilizing a single reference well (H7) of PBS-B containing no analyte for reference subtraction. Subtraction of the signal generated in H7 from A7–G7 corrects for sensor drift over time.

Panel B represents an assay utilizing parallel reference subtraction. Column 5 contains lipoparticles containing the target membrane protein CXCR4. Column 6 contains null lipoparticles. Parallel reference subtraction is executed by running the complete assay twice within one experiment. CXCR4 containing lipoparticles are used for the first pass (use of columns 1, 2, 3, 4, 5, 7, 8, 7) and null lipoparticles are used for the second pass (use of columns 1, 2, 3, 4, 6, 7, 8, 7). The signal generated by null particles is subtracted from the signal generated using CXCR4-containing particles, correcting for potential nonspecific interactions of the sample matrix with the lipoparticle-loaded biosensors.

Panel C represents an assay utilizing double reference subtraction (one reference well and one column of reference biosensors). Column 8 contains 7 wells with analyte (A8–G8) and one reference well with no analyte (H8). Column 6 contains null lipoparticles as reference biosensors. Similar to parallel reference subtraction, double reference subtraction is executed by running the complete assay twice within one experiment. CXCR4 containing lipoparticles are used for the first pass (use of columns 1, 2, 3, 4, 5, 7, 8, 7) and null lipoparticles are used for the second pass (use of columns 1, 2, 3, 4, 6, 7, 8, 7). Double reference subtraction corrects for both sensor drift over time and potential non-specific interactions between the sample matrix and lipoparticle loaded biosensors.

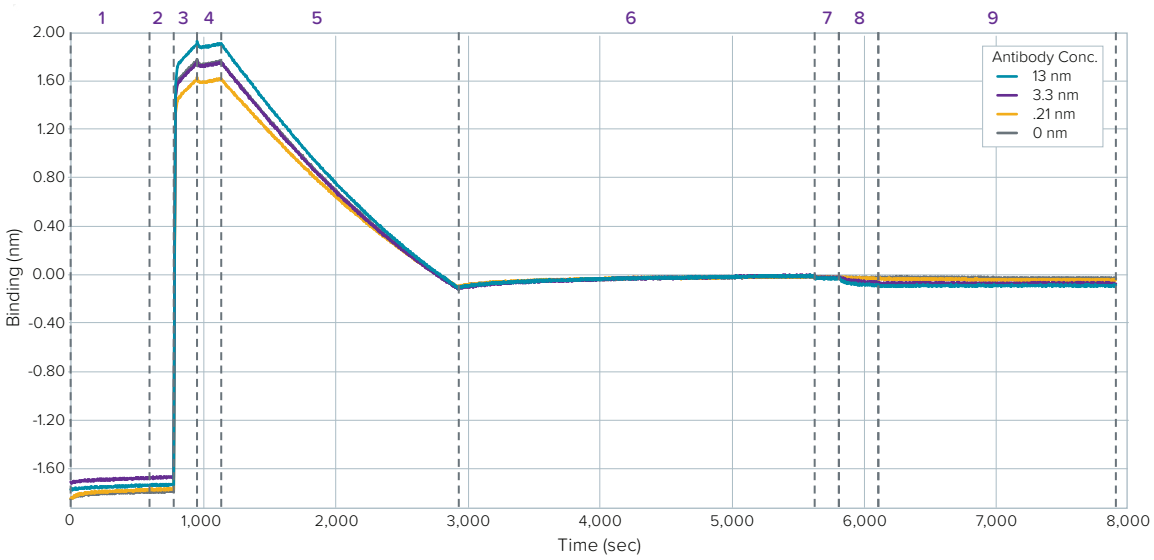


Figure 5: Complete kinetic experiment assaying the interaction between the lipoparticle-displayed membrane protein CXCR4 with an anti-CXCR4 antibody. Step 1: Equilibration, Step 2: Wash, Step 3: Loading of biotinylated wheat germ agglutinin on to a Streptavidin Biosensor, Step 4: Wash, Step 5: Loading of lipoparticle (negative amplitude upon binding due to size of the particle), Step 6: Wash, Step 7: Baseline, Step 8: Measure association of anti-CXCR4 antibody to lipoparticle-displayed CXCR4 Step 9: Measure dissociation of anti-CXCR4 antibody from lipoparticle-displayed CXCR4.

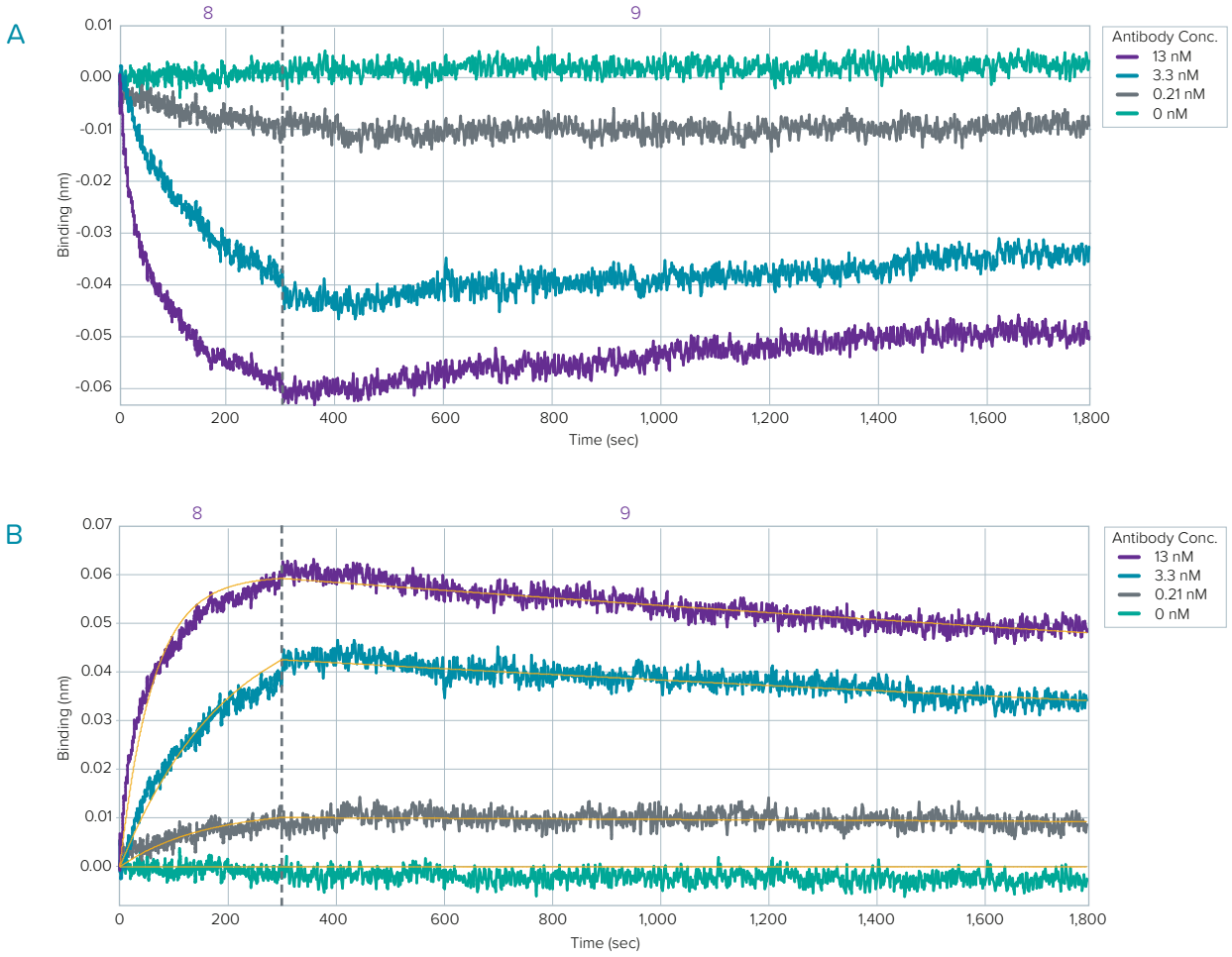
Step number	Step name	Time (seconds)	Shake speed	Step type
1	Equilibration	600	600	Custom
2	Wash	180	600	Custom
3	Load Biotin-WGA	180	600	Loading
4	Wash	180	600	Custom
5	Load CXCR4-Lipoparticles	1800	600	Loading
6	Wash	3500	600	Custom
7	Baseline	2700	600	Baseline
8	Association	300	1000	Association
9	Dissociation	1800	1000	Dissociation

Table 1: Assay method for kinetic analysis of the interaction between CXCR4-containing lipoparticles and an anti-CXCR4 antibody.

Due to its large size, the lipoparticle placed the interaction surface approximately 150 nm away from the biosensor surface. In this example, the extended distance caused the amplitude of observed data to be inverted, but otherwise identical (Figure 6A). The flip function of the Octet Data Analysis software was used to invert the signal. In the results table of the analysis tab, all rows were selected and flip was selected from the right-click menu. All data inverted so that the nm shift was transformed from negative to positive. Inversion of the data to a positive signal allowed curve fitting of both the association and dissociation steps with a 1:1 binding model using the global fitting function (grouped by color, Rmax unlinked by biosensor). Kinetic fitting results are displayed as red lines in Figure 6B and as quantitative values in Figure 6C. Specific processing settings can vary due to both the experimental setup and analyte-ligand system probed.

Validation of flip data software feature

A model system was developed to investigate the validity of inverting the kinetic data obtained from a lipoparticle experiment (Figure 7). In this model system, the interaction between a mouse anti-CXCR4 antibody and a donkey anti-mouse IgG was studied both on the surface of captured lipoparticles (resulting in inverted data) and on the surface of a Streptavidin Biosensor (standard data). The values of k_{on}, k_{diss}, and K_D, derived from curve fitting data for the two assays, were compared to determine if inverting the data from the lipoparticle assay delivered valid kinetic measurements.



K_D (nM)	k_{on} (1/Ms)	k_{on} Error (1/Ms)	k_{diss} (1/s)	k_{dis}s Error (1/s)
13.3	9.88 E+5	9.34 E+3	1.32 E-4	1.33 E-6

Figure 6: Expanded view of association (Figure 5, Step 8) and dissociation (Figure 5, Step 9) of lipoparticle-expressed CXCR4 and an anti-CXCR4 antibody. After correction of the signal using a reference biosensor, the processed data was negative and therefore not amenable to curve fitting (6A). Application of the Octet Data Analysis software flip function inverted the data (6B), allowing fitting with a 1:1 heterogeneous ligand fitting model (red lines) and derivation of quantitative kinetic data (6C).

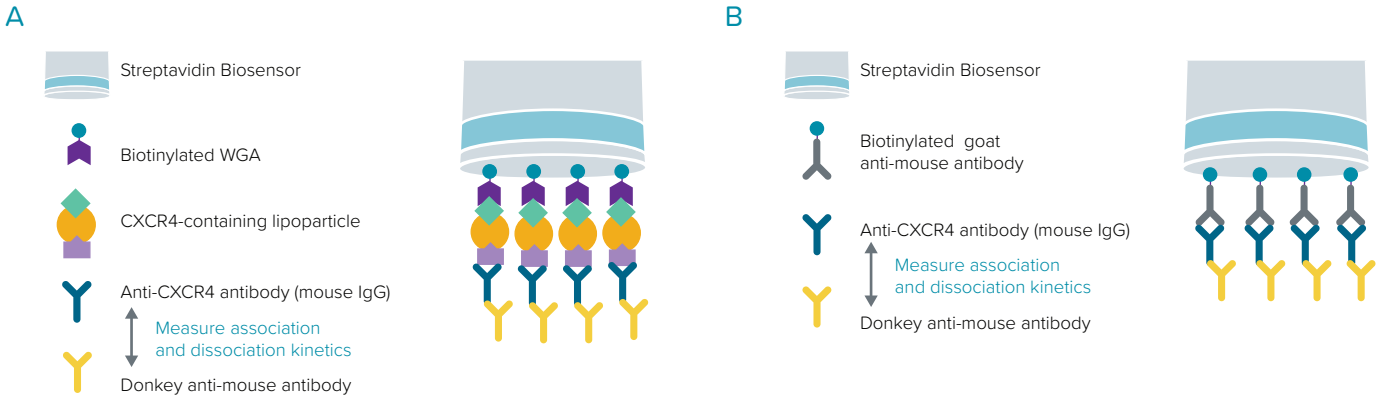


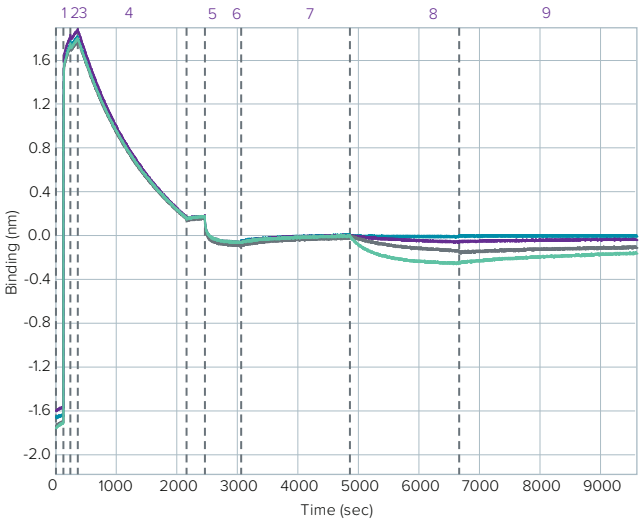
Figure 7: A model system developed to investigate the validity of inverting kinetic data obtained from a lipoparticle experiment. The interaction of the mouse anti-CXCR4 antibody with the donkey anti-mouse IgG antibody was replicated both with (7A) and without (7B) a lipoparticle. The experimental arrangement allowed the same molecular interaction to be analyzed as inverted data (from a lipoparticle, 7A) and non-inverted data (no lipoparticle, 7B).

The lipoparticle-based model system was evaluated in a 8-step assay (Figure 8A). After biosensor equilibration (Figure 8A, Step 1), biotinylated WGA (50 µg/mL) was loaded onto the Streptavidin Biosensor (Figure 8A, Step 2). After a brief washing step (Figure 8A, Step 3), WGA was used to capture the lipoparticle displaying CXCR4 out of solution (10 µg/mL) (Figure 8A, Step 4), producing a large negative shift due to the binding of the 150 nm particle. After a wash step (Figure 8A, Step 5), the mouse anti-CXCR4 antibody (5 µg/mL) was loaded onto the surface of the lipoparticle (Figure 8A, Step 6). A baseline was established (Figure 8A, Step 7) and the interaction of interest, the binding of a donkey anti-mouse antibody with the mouse anti-CXCR4 antibody, was then observed (Figure 8A, Steps 8 and 9). The binding of the donkey anti-mouse antibody to the anti-CXCR4 antibody was measured in a half-log titration series from 3 to 100 nM, with the 3 nM and 100 nM samples run in duplicate. A 0 nM donkey anti-mouse IgG sample probed with CXCR4-containing lipoparticles was used as a reference control. The reference corrected data was inverted with the flip feature of the Octet Data Analysis software and fit to a 1:1 kinetic binding model (Figure 8B) to obtain values for k_{on}, k_{diss} and K_D (Figure 8C) as described in the Data Processing section.

The second model system assay, arranged without a lipoparticle to produce binding data with a positive nm shift, was performed in a 7-step assay (Figure 8D). After Streptavidin Biosensor equilibration (Figure 8D, Step 1), biotinylated goat anti-mouse antibody (0.63 µg/mL) was loaded (Figure 8D, Step 2). Excess goat anti-mouse antibody was removed by washing (Figure 8D, Step 3) and mouse anti-CXCR4 antibody (5 µg/mL) was loaded (Figure 8D, Step 4). Excess mouse anti-CXCR4 was removed by washing (Figure 8D, Step 5) and the interaction of interest was then studied by binding donkey anti-mouse antibody to the mouse anti-CXCR4 antibody in a half-log titration (Figure 8D, Step 6). The donkey anti-mouse antibody was bound in a half-log titration series from 3 to 100 nM. Dissociation of the donkey anti-mouse antibody from the mouse anti-CXCR4 antibody was observed for one hour in PBS-B. A zero nM donkey anti-mouse antibody sample probed with a biosensor loaded with goat anti-mouse and mouse anti-CXCR4 antibodies was used as a reference control. The reference-corrected data was fit to a 1:1 kinetic binding model (Figure 8E) to obtain values for k_{on}, k_{diss} and K_D (Figure 8F) as described in the Data Processing section.

After reference subtraction, data for both systems was successfully fitted using a 1:1 binding model to obtain values for kon, k_{diss} and K_D (Figures 8C and 8F). The k_{on} values obtained with (1.83 E+4 M-1s-1) and without (1.26 E+4 M-1s-1) the lipoparticle agreed within 0.57 E+4 M-1s-1. The k_{diss} values with (1.42 E-4s-1) and without (7.13 E-5s-1) the lipoparticle agreed within 0.7 E-4s-1. The K_D values, calculated as the ratio of k_{diss}/k_{on}, differed by less than 2.1 nM. Raw binding data for both experiments produced concentration-dependent binding curves for analyte (donkey anti-mouse antibody) from 3 to 100 nM (compare Figures 8B and 8E). While the total nm shift observed for the model system without the lipoparticle was consistently greater than corresponding data in the presence of the lipoparticle, derivation of kinetic values is independent of the magnitude of the nm shift and the difference therefore does not bear relevance to the experimental objective of obtaining kinetic information. Comparison of the data for model system experiments performed in the presence and absence of the lipoparticle therefore demonstrates excellent agreement between the two results, validating kinetic characterization of membrane protein-ligand interactions on a lipoparticle using the ForteBio Octet platform.

A



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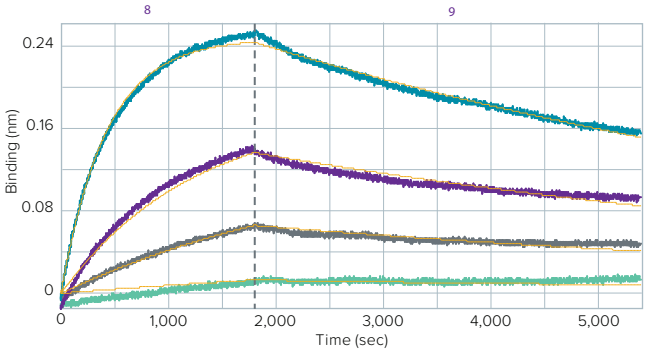
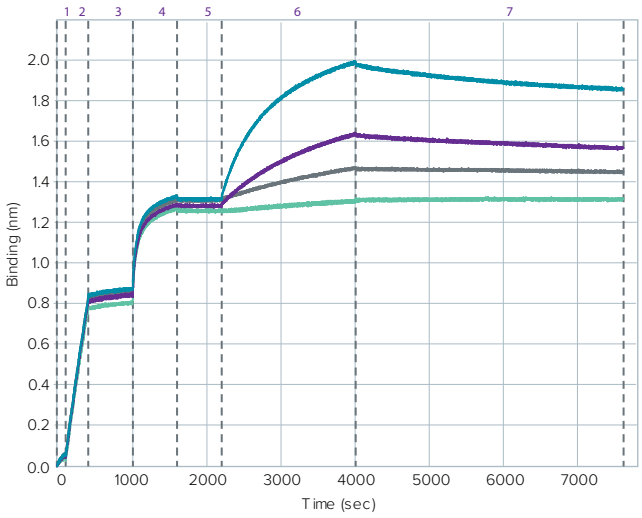


C

K_D (nM)	k_{on} (1/Ms)	k_{on} Error (1/Ms)	k_{diss} (1/s)	k_{diss} Error (1/s)
7.78	1.83 E+4	2.41 E+1	1.42 E-4	2.59 E-7

D



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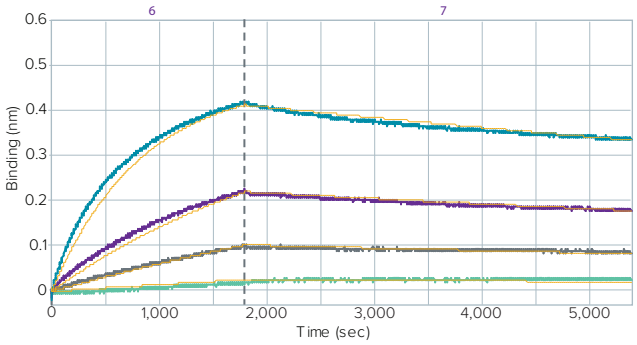

F

K_D (nM)	k_{on} (1/Ms)	k_{on} Error (1/Ms)	k_{diss} (1/s)	k_{diss} Error (1/s)
5.68	1.26 E+4	1.08 E+1	7.13 E-5	1.42 E-7

Figure 8: Example data from a model system designed to compare inverted and non-inverted kinetic data. The interaction between a mouse anti-CXCR4 antibody and a donkey anti-mouse antibody was measured both with (8A, 8B) and without (8D, 8E) the presence of a lipoparticle. The interaction between a mouse anti-CXCR4 antibody and a donkey anti-mouse antibody was measured on the surface of a lipoparticle in a 9-step assay (8A): Step 1: Streptavidin Biosensor equilibration, Step 2: Load biotinylated WGA, Step 3: Wash, Step 4: Load lipoparticle, Step 5: Wash, Step 6: Load mouse anti-CXCR4 antibody, Step 7: Baseline, Step 8: Measure association of donkey anti-mouse FAb to mouse anti-CXCR4 antibody, Step 9: Measure dissociation of donkey anti-mouse FAb from mouse anti-CXCR4 antibody. The association and dissociation steps from 8A were inverted for processing (8B). Curve fitting to the inverted data provided k_{on}, k_{diss}, and K_D for the interaction on a lipoparticle (8C). The interaction between a mouse anti-CXCR4 antibody and a donkey anti-mouse FAb fragment was measured without a lipoparticle in a 7-step assay (8D): Step 1: Streptavidin Biosensor equilibration, Step 2: Load biotinylated anti-mouse FAb, Step 3: Wash, Step 4: Load mouse anti-CXCR4 antibody, Step 5: Wash, Step 6: Measure association of donkey anti-mouse FAb to mouse anti-CXCR4 antibody Step 7: Measure dissociation of donkey anti-mouse FAb from mouse anti-CXCR4 antibody. The association and dissociation steps from 8D were processed and fit with a 1:1 binding model (8E). Curve fitting of the processed curves provided k_{on}, k_{diss}, and K_D for the interaction without a lipoparticle (8F).

FORTÉBIO

www.fortebio.com

ForteBio
47661 Fremont Boulevard
Fremont, CA 94538
888.OCTET-75 or 650.322.1360
fortebio.info@moldev.com

ForteBio Analytics (Shanghai) Co., Ltd.
No. 88 Shang Ke Road
Zhangjiang Hi-tech Park
Shanghai, China 201210
salesops.china@moldev.com

Molecular Devices (UK) Ltd.
660-665 Eskdale
Winnersh Triangle
Wokingham, Berkshire
RG41 5TS, United Kingdom
+44 118 944 8000
uk@moldev.com

Molecular Devices (Germany) GmbH
Bismarckring 39
88400 Biberach an der Riss
Germany
+ 00800 665 32860

AN-4003 Rev C