FOx BIOSYSTEMS
Breaking limits in bioanalysis

WHITE PAPER

Kinetic analysis of V_HH and antibodies with WHITE FOx

White paper 10 | Version 1 | Tobias Zbik, Sarah Parmentier, Kris Ver Donck

Abstract

The landscape of antibody research continuously evolves, with novel antibody variants such as V_HH, antibody-drug conjugates, and bivalent antibodies emerging with huge potential in drug development. This white paper explores the kinetic analysis of V_HH and a human bivalent antibody using FO-SPR technology. We showcase how the remarkable sensitivity of the SPR dip-in method enables the measurement of high-affinity kinetics, even for low molecular weight antibodies like V_HH. Furthermore, real-time measurements and flexibility in assay design provide a means to address challenging experimental designs, particularly relevant for studying bivalent antibodies.

Introduction

Antibodies have long been the cornerstone in the fields of life science, medicine, and biotechnology, enabling the development of diagnostics and therapeutics, and serving as valuable research agents. The emergence of new antibody variants, such as single-domain antibodies (sdAbs) and bivalent antibodies, has significantly advanced the field of antibody research by offering innovative solutions to longstanding challenges while expanding the repertoire of tools available for scientific inquiry and therapeutic development.

While highly effective, traditional antibodies often come with limitations such as large size, complex structure, and difficulties in discovery, development, and production. sdAbs have emerged as a promising class of antibody fragments offering unique advantages over their conventional counterparts. sdAbs, also known as Nanobodies[1] or V_HH antibodies, are derived from the variable regions of heavy-chain-only antibodies naturally found in camelids and cartilaginous fish and naturally target one singular epitope. Their remarkably small size, robust stability, and exceptional binding affinity make them invaluable tools in research, diagnostics, and therapeutics.

In the fields of antibody engineering and therapeutic development, bivalent antibodies are a compelling class of biologics applied in precision medicine and targeted therapy. Bivalent antibodies are capable of binding two identical antigen-binding sites, enabling enhanced target engagement and effector function. This binding mechanism naturally leads to increased avidity and improved affinity compared to monovalent antibodies.

WHITE FOx offers a valuable alternative to conventional SPR as a convenient benchtop instrument that combines the performance of SPR with much-reduced complexity. The fiber-optic probes can simply be immersed in the sample of interest (Figure 1). This approach eliminates the contamination and clogging issues often encountered in microfluidic chips when analyzing crude samples like serum, plasma, or whole blood.

This study demonstrates the application of dip-in sensing to analyze the binding kinetics of single-domain and bivalent antibodies, providing a rapid and straightforward setup that yields precise data on high-affinity interactions. Initially, we elucidate the interaction between anti-rabbit IgG V_HH and rabbit IgG using the one-to-one kinetic model. Subsequently, we explore the binding of the bivalent antibody mAb2 to its dimeric target AgX using the bivalent kinetic model.

These case studies underscore the efficacy and adaptability of FO-SPR technology in the dynamic landscape of antibody development.

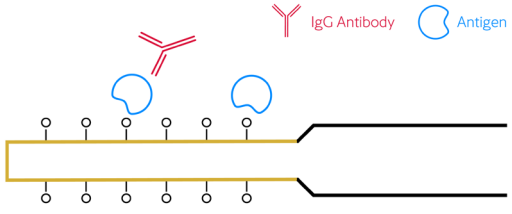

Figure 1: Dip-in sensing for characterization of antibody-antigen binding interaction

[1] Nanobodies is a registered trademark of Sanofi

Materials and methods

Dip-in sensing

FOx BIOSYSTEMS turned an optical fiber into a mass-sensitive sensor using the well-established SPR principle for biomolecular interaction studies. The SPR effect is achieved by coupling a white light source to the fiber optic sensor probe. The FO-SPR sensor is coated with bioreceptor molecules that can bind to the target molecules of interest. This binding results in a real-time wavelength shift that produces a sensor signal, as shown in Figure 2. The sensor probe is simply dipped into the liquid sample to measure biomolecular interactions directly.

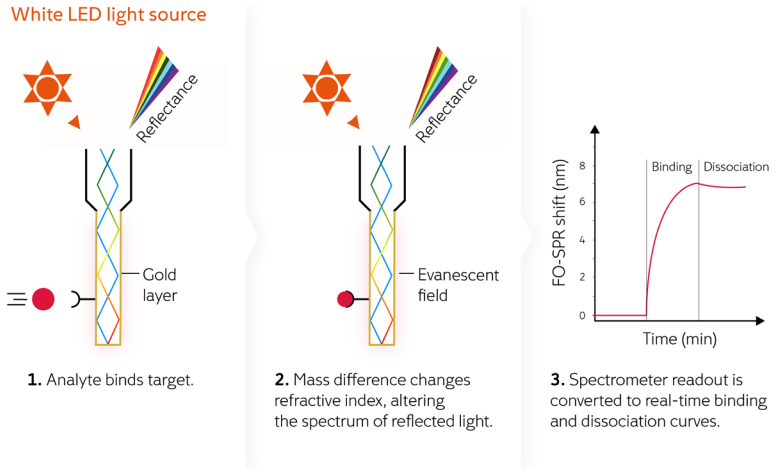

Figure 2: The FO-SPR principle

Tools and reagents

Buffers / reagents	Concentration
Anti-rabbit IgG V_HH	5 µg/ml in MEST
Rabbit IgG	10 – 40 nM in PBST
AgX-biotin	20 µg/ml in PBST
mAb2	0.4 - 100 nM in PBST
WHITE FOx carboxyl sensor probes	-
WHITE FOx streptavidin sensor probes	-
PBS(T)	1X, 0.01% Tween-20, pH 7.4
MES(T)	10 mM MES, 0.01% Tween-20, pH 5.7
EtAm: Ethanolamine	0.05 M, pH 8.5
EDC: 1-ethyl-3-[3-dimethylaminopropyl] carbodiimide	0.4 M in EDC/NHS
NHS: N-hydroxysuccinimide	0.1 M in EDC/NHS

Table 1: Buffers and reagents

Kinetic analysis of rabbit IgG binding to anti-rabbit IgG V_HH

To showcase the capabilities of WHITE FOx in the analysis of V_HH binding, we investigated the interaction between anti-rabbit IgG V_HH and rabbit IgG. The methods in this section build on the probe surface chemistry optimization for carboxyl probes found in white paper 1: "Carboxyl probe immobilization for label-free protein quantification." For the immobilization of anti-rabbit IgG V_HH, carboxyl probes were hydrated in MEST buffer and subsequently activated for 5 minutes in EDC/NHS, followed by a washing step in MEST buffer. The activated probes were dipped for 500 seconds into the anti-rabbit IgG V_HH solution (5 µg/ml in MEST buffer) to covalently immobilize the IgG on the carboxyl surface.

The immobilization was followed by a 5-minute quenching step in EtAm and a washing step in PBST. To determine the association rate, the carboxyl probes with immobilized anti-rabbit IgG V_HH were exposed to a concentration series of rabbit IgG (40 nM – 10 nM in PBST) for 400 seconds. Dissociation was induced by transferring the probes to a fresh well containing PBST for 600 seconds. All assay steps were conducted under a controlled sample temperature of 26°C and a shaking speed of 1000 rpm.

Kinetic analysis of mAb2 binding to AgX

We analyzed the sensorgram data from the binding experiments with the TraceDrawer kinetic analysis software using the general one-to-one kinetic model for monovalent binding and the bivalent model for bivalent binding to a single antigen.

Here, we demonstrate the binding of the bivalent antibody mAb2 to its dimeric target AgX. Streptavidin probes were hydrated in PBST and subsequently dipped for 180 seconds into an AgX-biotin solution (2 µg/ml in PBST) to immobilize the AgX on the probe surface via the streptavidin-biotin interaction. The immobilization was followed by a 600-second washing step in PBST.

The association rate was analyzed by dipping the sensors for 300 seconds into a concentration series of mAb2 solutions, ranging from 1.56 - 25 nM in PBST. Dissociation was induced by transferring the probes to a fresh well containing PBST for 300 seconds. All assay steps were conducted with the sample temperature controlled at 26°C and a shaking speed of 1000 rpm.

Results

Kinetic analysis of anti-rabbit IgG V_HH binding to rabbit IgG

The immobilization of anti-rabbit IgG V_HH on the carboxyl sensor surface was followed on WHITE FOx. The relatively small V_HH (15 kDa) showed an association curve and reached saturation at around 4.5 ± 0.2 nm shift (Figure 3).

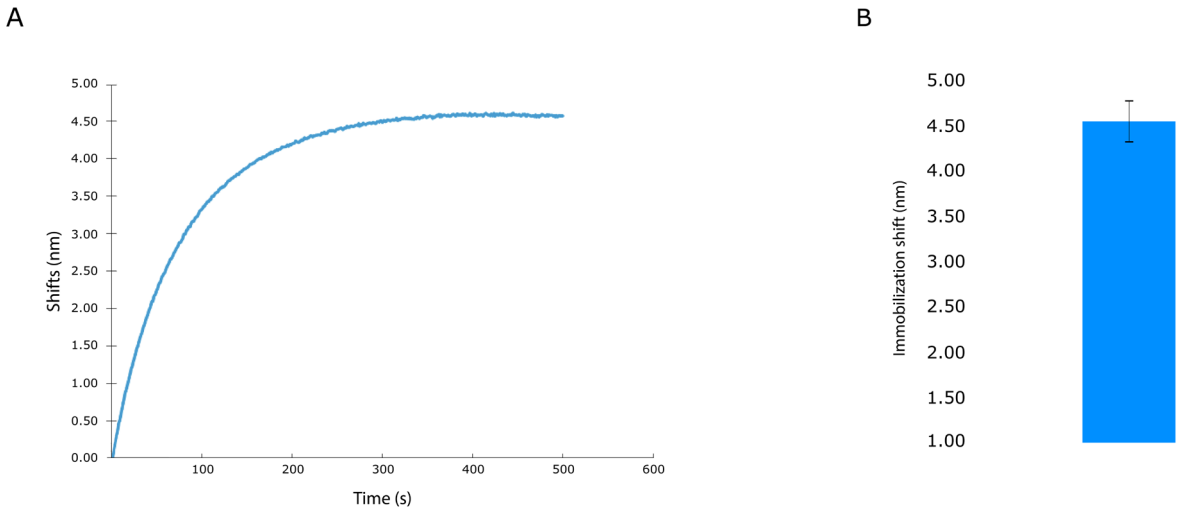

Figure 3: Immobilization of anti-rabbit IgG V_HH on carboxyl probes
A. Representative immobilization diagram.
B. Immobilization shift levels of multiple measurements (n=12)

Next, to observe the association with the immobilized V_HH, the sensors were immersed in a series of rabbit IgG solutions with concentrations ranging from 10 to 40 nM in PBST. The varying concentrations exhibited distinct association curves, with the highest concentration resulting in a shift of up to 3 nm (Figure 4). To follow dissociation, the probes were transferred to fresh PBST. Kinetic parameters (k_{on}, k_{off}, and k_D) were determined using a fitting procedure in TraceDrawer software. A global fit employing a one-to-one binding model was utilized to obtain the kinetic parameters detailed in Table 2.

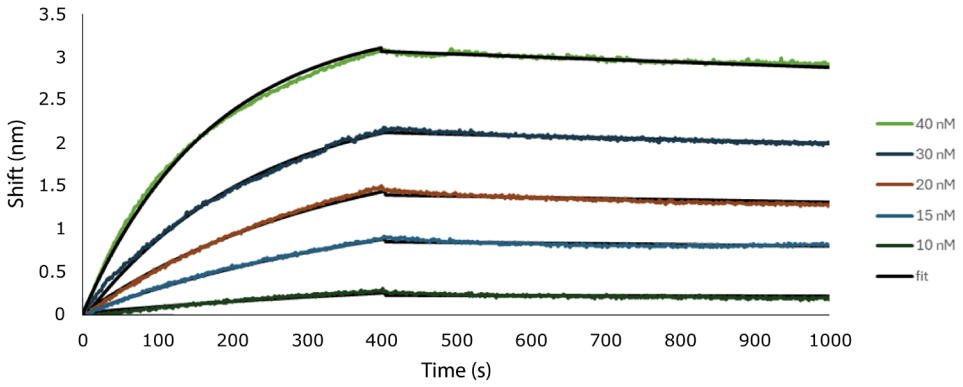

Figure 4: Kinetic analysis of anti-rabbit IgG V_HH binding to rabbit IgG Association and dissociation steps with the corresponding fit derived from a one-to-one binding model in a global fit.

k_{on} (1/(M*s))	k_{off} (1/s)	k_D (M)	7.26E-10	U-Value (k_D %)
1.43E+05	1.04E-04	7.26E-10	0.0001	3.1

Table 2: Kinetic parameters of anti-rabbit IgG V_HH binding to rabbit IgG. Control values confirm a very good fit between the experimental data and the one-to-one model.

Kinetic analysis of bivalent antibody

The biotinylated dimeric target AgX was immobilized on streptavidin probes. At a target concentration of 20 µg/ml, an association shift of around 2.5 ± 0.2 nm was observed (Figure 5). At this concentration, the signal reached saturation, indicating that the probe surface was densely packed with AgX molecules.

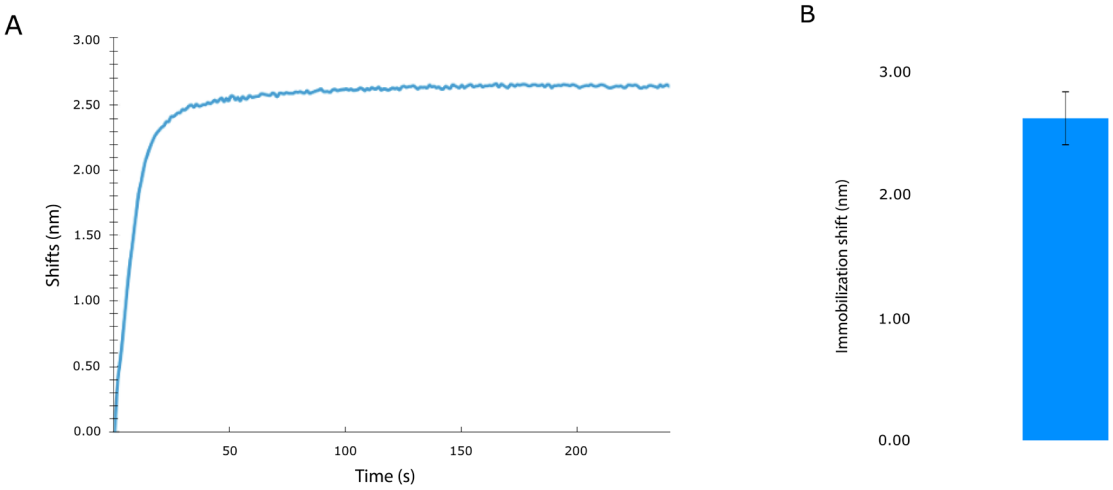

Figure 5: Immobilization of AgX on streptavidin probes
A. Representative immobilization diagram.
B. Immobilization shift levels of multiple measurements (n=12)

Following this, the sensors were dipped in a set of mAb2 solutions, featuring concentrations spanning from 1.56 – 25 nM in PBST, for the observation of the association step. The different concentrations displayed discernible association rates, with the maximum concentration inducing a shift of up to 0.4 nm (Figure 6). For dissociation analysis, the probes were moved to fresh PBST. Kinetic parameters (k_{on}, k_{off}, and k_D) were calculated through a fitting process using TraceDrawer software. A global fit employing a bivalent binding model yielded the kinetic parameters outlined in Table 3.

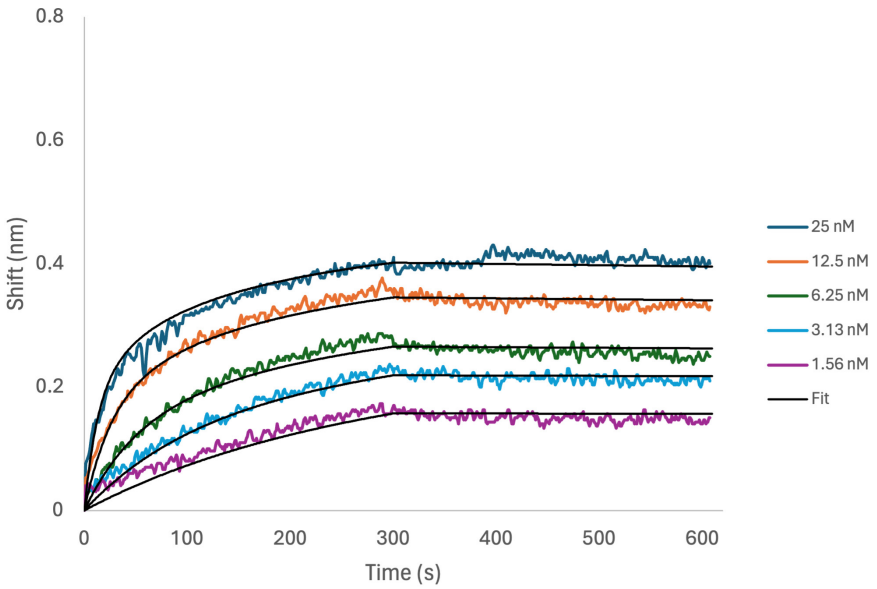

Figure 6: Kinetic analysis of mAb2 binding to AgX association and dissociation steps with the corresponding fit derived from a bivalent binding model in a global fit.

$k_{on}1$ (1/(M*s))	$k_{off}1$ (1/s)	k_D1 (M)	$k_{on}2$ (1/(Signal*s))	$k_{off}2$ (1/s)	k_D2 (M)	Chi^2 ([Signal(nm)]^2)
1.36E+06	5.97E-05	4.39E-11	1.01E+00	4.28E-02	4.26E-02	0.0002

Table 3: Kinetic parameters of mAb2 binding to AgX

Discussion

This white paper showcases the efficacy of FO-SPR in antibody kinetics applications, particularly its utility in determining the kinetic parameters of V_HH and bivalent antibodies.

A significant challenge when working with V_HH lies in their small size and short distance from the sensing surface, which complicates detection using kinetic methods, coupled with their high affinities, making it difficult to obtain reliable kinetic data. Here, we have demonstrated that WHITE FOx can effectively detect V_HH immobilization on carboxyl probes, thanks to the exceptional sensitivity of FO-SPR technology. Moreover, we successfully investigated a high-affinity interaction and derived kinetic parameters from it. This highlights WHITE FOx as a valuable tool for analyzing V_HH and other small antibody fragments with high affinities.

Bivalent antibodies present a challenge due to the very high-affinity interaction with their targets, characterized by a very slow dissociation rate, coupled with avidity effects stemming from their dual binding sites. WHITE FOx yielded a full kinetic analysis of high-affinity interactions over a broad concentration range.

Furthermore, the streptavidin probes offered a fast and easy method to derive the kinetic data starting from a biotin-conjugated target. Although careful experimental design remains crucial for the success of such measurements, the flexibility and high sensitivity of WHITE FOx assists in optimizing the experimental setup and helps to derive quality data sets even for high-affinity interaction pairs.

Antibody variants such as V_HH and bivalent antibodies represent substantial progress in antibody research but come with challenges for their characterization. Models such as one-to-one interaction provide powerful insight into the kinetic characteristics of these antibodies, but the presence of multiple binding sites, either in the antibody or their target, may require more specific models, such as the bivalent interaction, for detailed kinetic characterization.

WHITE FOx presents a potent solution, merging the high sensitivity of SPR with the easy-to-use dip-in method. This tool equips scientists to effectively address the evolving challenges in kinetic analysis across the diverse range of antibody variants prevalent in modern life sciences.

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