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Breaking limits in bioanalysis

WHITE PAPER

Affinity determination of single domain antibodies for detection of biomarkers

White paper 8 | Version 2 | Laura Marin, Sarah Parmentier, Kris Ver Donck, Filip Delport

Abstract

Single domain antibodies (sdAbs) and adhirons are an alternative to conventional antibodies for diagnosis purposes, thanks to their improved capacity for penetrating into tissues. These molecules are currently selected using ELISA. However, this technique does not provide all the kinetic information that is crucial for choosing better binders. To overcome this, we propose the use of FOx BIOSYSTEMS' fiber-optic surface plasmon resonance technology (FO-SPR). It is a robust tool that harnesses the power of SPR in an easy-to-use dip-in fiber-optic configuration.

This document describes how to use WHITE FOx to determine the best binder for a specific biomarker. As an example, we used the monomeric form of C-reactive protein (CRP), a protein secreted by the liver that is widely used as a non–specific marker to monitor the development of infection and inflammation. The ability to bind this protein by different single domain antibodies and adhirons was evaluated and kinetics parameters such as association and dissociation constants were also calculated.

The method shown here demonstrates that FO-SPR technology can provide label-free, real-time, fast kinetic characterization, and is superior to traditional assays that can only give end point data.

Introduction

Antibodies are commonly used binding proteins that are extremely important in scientific research, diagnostic and therapy. Nevertheless, they have a number of limitations, including ineffective tissue penetration or incorrect folding, which makes them inadequate for certain applications. To overcome these difficulties, alternative binding proteins are used: sdAbs and adhirons.

Single domain antibodies, also referred to as nanobodies[1], are recombinantly produced antigen-binding V_HH fragments that are derived from the heavy chain only antibodies common in a few animal species, such as camelids and sharks.

Adhirons are small (~12 KDa) non-antibody binding proteins. They comprise a genetically engineered consensus plant phytocystatin protein sequence, and two regions with variable sequence that provide the binding interface.

An important step in the production of both sdAbs and adhirons is screening the libraries to select the best binders. Traditionally, the detection method used is enzyme-linked immunosorbent assay (ELISA). However, this method is time consuming and only shows endpoint data, missing important information such as association and dissociation rates. Surface plasmon resonance (SPR), a powerful biosensing technology that offers label-free real-time measurement, offers a suitable alternative. SPR exploits electromagnetic waves to measure local refractive index changes caused by suspended analytes and immobilized biomolecules on the sensor surface. Despite being sensitive and reliable biosensors, they typically use microfluidics, making them neither appropriate for quick testing because of the size and cost of the instruments, nor for crude samples due to clogging.

An alternative to microfluidics-based SPR is fiber-optic surface plasmon resonance (FO-SPR). Here, a gold layer is applied externally to fiber-optic SPR sensor probes which can be dipped into the sample of interest, enabling fast testing, easy to use protocols and minimal processing. Moreover, there is no risk of clogging and associated contamination that can occur with classical SPR. Thanks to this, measurements can be performed directly in crude biological samples, as demonstrated before (white paper 2).

[1] NANOBODY is a registered trademark of Sanofi (Ablynx N.V.)

In this white paper, we describe how to determine the best binder of a biomarker through kinetics analysis using FO-SPR technology (Figure 1). As an example, we immobilized C-reactive protein (CRP), a non-specific diagnostic marker of inflammation, on generic carboxyl probes. We then bound one sdAb and one adhiron anti–CRP to the immobilized CRP to characterize the kinetics of the binding interaction.

Materials and methods

What is FO–SPR

FOx BIOSYSTEMS turned an optical fiber into a mass sensitive sensor using the well-established surface plasmon resonance (SPR) principle for biomolecular interaction studies. The SPR effect is achieved by coupling a white light source to the fiber optic sensor probe.

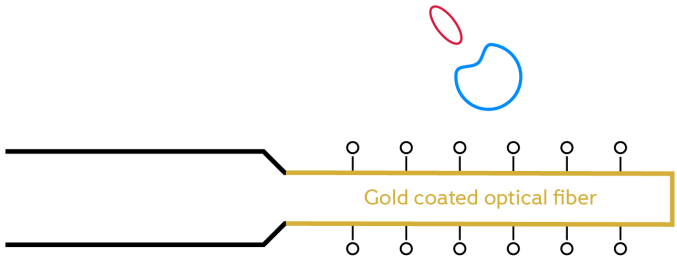
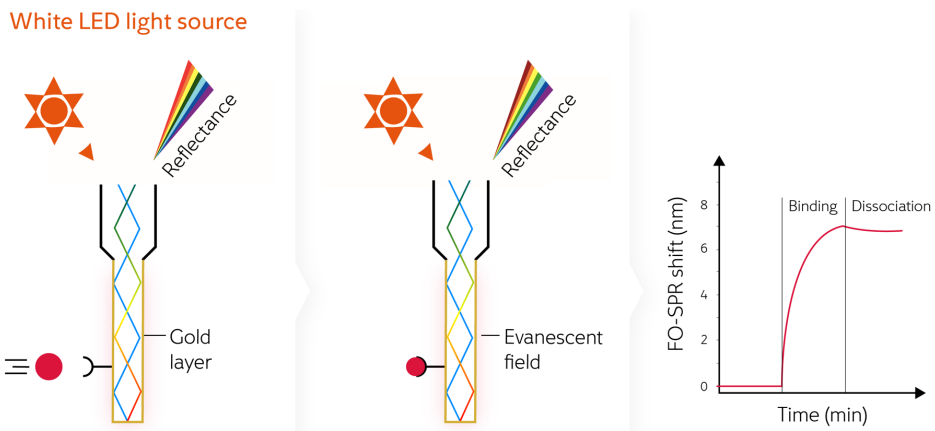


Figure 1: Representation of a carboxyl sensor probe with immobilized protein binding to an sdAb.

In the probe, the light interacts with a gold layer and senses the refractive index up to 200 nm away from the outer surface. At the tip of the probe, light is reflected back through the bifurcated fiber to a spectrometer. The resonance condition is monitored by tracking the wavelength at which the least light is reflected. The fiber-optic surface plasmon resonance (FO-SPR) sensor is coated with bioreceptor molecules which can bind to the target molecules of interest. This binding changes the refractive index, resulting in a wavelength shift that produces a sensor signal as shown in Figure 2: FO-SPR principle. The sensor probe is simply dipped into the liquid sample to measure biomolecular interactions directly.



1. Analyte binds target.

2. Mass difference changes refractive index, altering the spectrum of reflected light.

3. Spectrometer readout is converted to real-time binding and dissociation curves.

Figure 2: The FO-SPR principle

Tools and reagents

For the procedures in this white paper you will need:

- WHITE FOx instrument with FOx-SPR acquisition software and the FOx data processing tool.
- TraceDrawer or other software for kinetics evaluation.
- Microsoft Excel or other data calculation software for data handling and viewing.
- Carboxyl probes.
- Micro pipettes from 10 to 1000 µl with disposable tips.
- A 96-well PCR plate or 8-well PCR strips.
- On-desk refrigeration for protein solutions.
- LoBind Eppendorf tubes for protein solutions.
- A microtube mixer and mini centrifuge to remove protein aggregates.

Buffers / reagents	Concentration
MES: 2-(N-morpholino) ethanesulfonic acid pH 6.0	50 mM
1-ethyl-3-[3-dimethylaminopropyl] carbodiimide (EDC)	0.4 M in EDC/NHS
N-hydroxysuccinimide (NHS)	0.1 M in EDC/NHS
Ethanolamine-HCl, pH 8.5 (EtAm)	50 mM
Capture molecule (CRP)	10 µg/ml final concentration
Target molecules (sdAb and adhirons)	Up to 533.33 nM final concentration.
Bovine serum albumin (BSA)	1%
NaOAc+ 0.01% Tween pH 5.0	10 mM
PBS + 0.01% Tween pH 7.2	10 mM

Table 1: Reagents and buffers.

Note: Special attention is required for EDC/NHS which is a highly reactive and short-lived reagent (EDC user guide, Sigma Aldrich) and must be prepared just prior to use.

Surface functionalization of FO probes with the antigen CRP

Carboxyl probes were hydrated in MES buffer prior to activation. Carboxyl groups were activated by incubation in 0.4 M EDC/0.1 M NHS in MES buffer for 5 minutes. A 10 µg/ml solution of CRP in 10 mM NaOAc 0.01% Tween at pH 5.0 was covalently immobilized to the activated carboxyl groups for 5 minutes. EtAm quenching was used to stop the immobilization and a further MES buffer wash removed non-covalently bound proteins. One probe with no CRP was included as a negative control. All protocol run steps were performed at 26°C with shaking at 1000 rpm.

sdAb binding

sdAb anti-CRP was coupled with the functionalized probes at increasing concentrations (two-fold serial dilutions from 8.33 nM to 133 nM in 10 mM PBS buffer with 0.01% Tween, pH 7.2) on a single probe. The experiment was performed on two separate probes with immobilized CRP and control with no immobilized protein to test non-specific binding. Association and dissociation steps were measured for 120 seconds each.

Adhiron binding

Adhiron anti-CRP was coupled with the functionalized probes at increasing concentrations (two-fold serial dilutions from 13.51 nM to 216.22 nM in 10 mM PBS buffer with 0.01% Tween and 0.05% BSA at pH 7.2) on a single probe in triplicate. This was also performed on control with no immobilized protein. Association and dissociation steps were measured for 300 seconds each.

Results

Non – specific binding controls

Both sdAb and adhiron anti-CRP show some non-specific binding to the control probe with no immobilized protein. However, signals are much smaller and clearly distinguishable from specific binding as observed in Figure 3. If required, non-specific binding could be further reduced by optimizing assay conditions or immobilizing an irrelevant protein to the blank control probes.

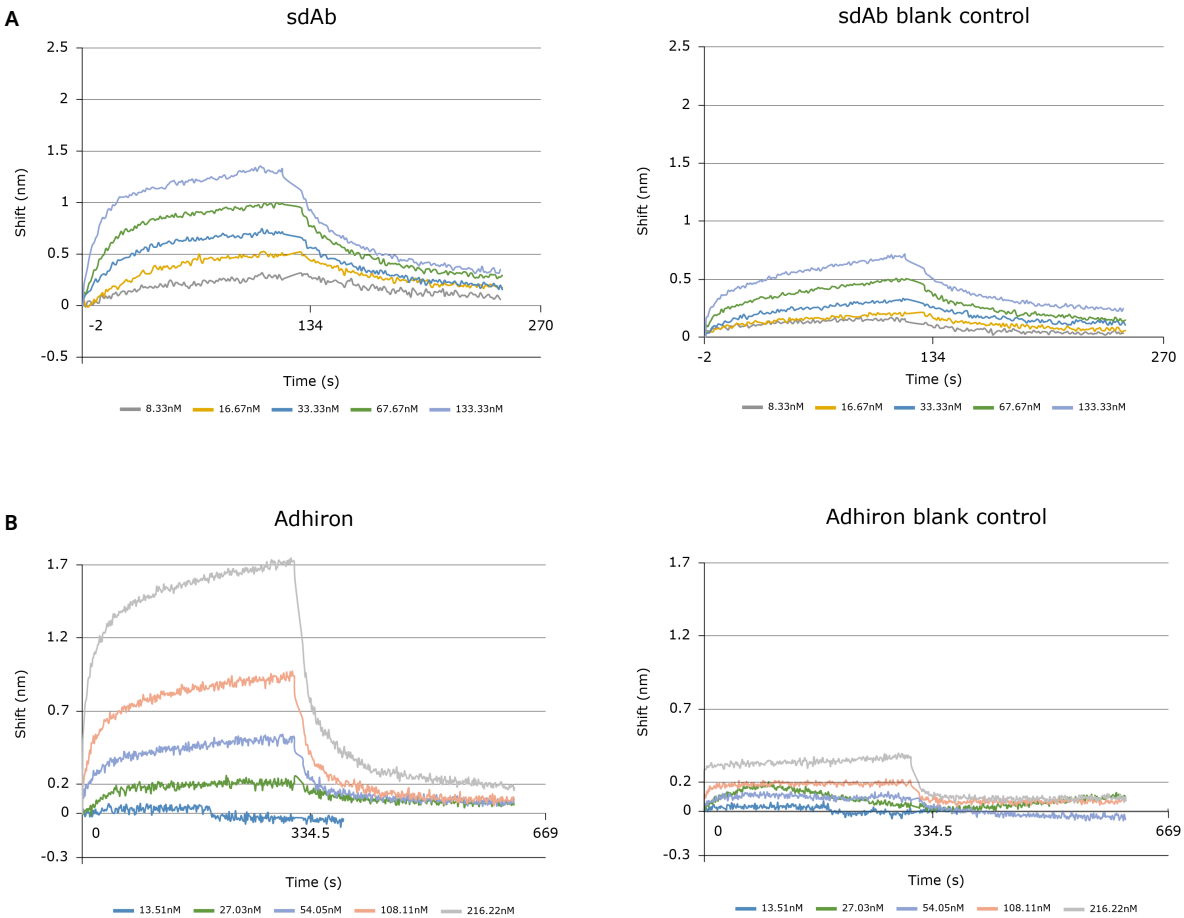


Figure 3: Binding of sdAb anti-CRP (A) and of adhiron anti-CRP (B) to functionalized probe (left) and to control probe with no immobilized protein (right).

Kinetic FO-SPR analysis

In order to determine the kinetic binding parameters (K_{on}, K_{off} and K_D), five concentrations of sdAb and of adhiron anti-CRP were measured and later analyzed with the software TraceDrawer. The one-to-one binding kinetic model was chosen for the analysis. The K_{on}, K_{off} parameters were set as global to obtain more robust results and Bmax was set as local.

The obtained kinetic parameters are indicated in Table 2 and the fitted model shown in Figure 4.

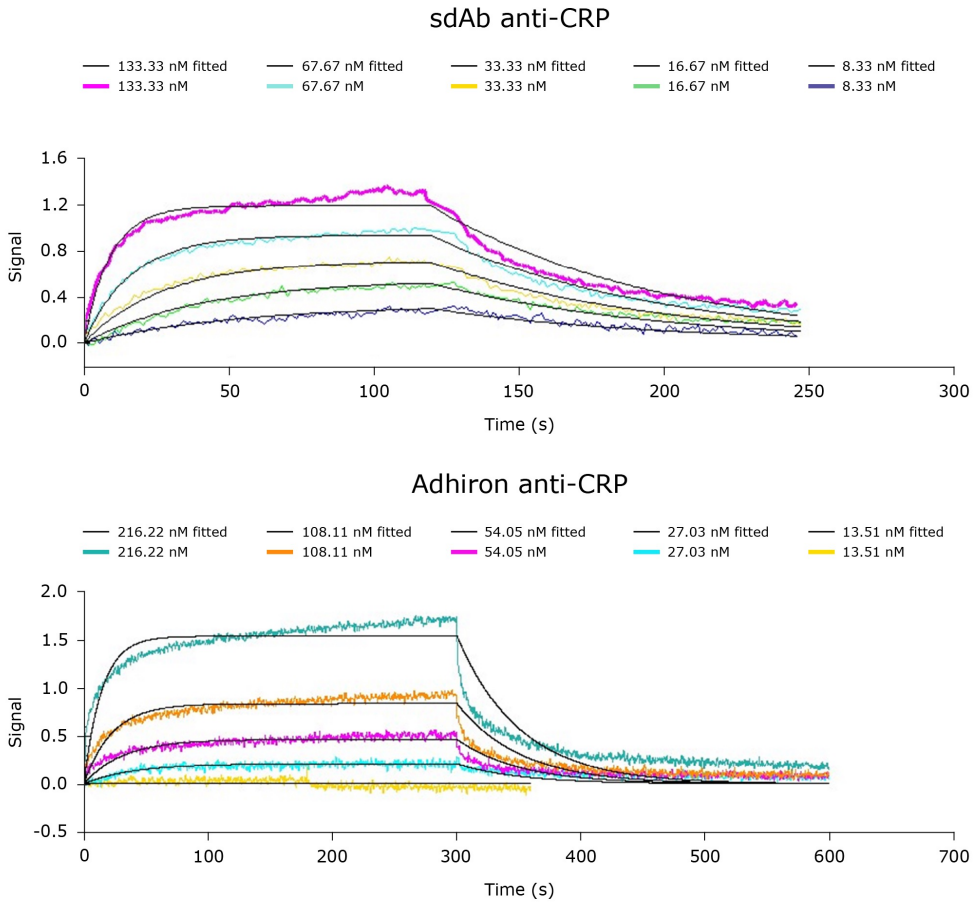


Figure 4: Fit data of sdAb and adhiron anti-CRP obtained by analysis with TraceDrawer.

	K_{on} (1/(M*s))	K_{off} (1/s)	K_D (M)	Chi^2	U-value (%)
sdAb 1	7.21E+05	1.27E-02	1.76E-08	0.00	1,70
sdAb 2	7.27E+05	1.67E-02	2.29E-08	0.01	4,30
Adhiron	2.35E+05	1.97E-02	8.47E-08	0.01	6,70

Table 2: Kinetic binding parameters of sdAb (two separate measurements) and adhiron anti-CRP obtained by analysis with TraceDrawer.

Conclusions

To identify the best binder to a target molecule, it is especially important to determine all kinetics parameters for each of the candidate interacting pairs: K_D, K_{on}, and K_{off}. These values describe the interaction between two partners in the binding equilibrium and is a measure of their affinity and is considered a crucial parameter to compare different ligands.

In this dataset, the adheron shows a higher K_D, which is mainly contributed to by a lower K_{on} than the sdAbs tested, indicating faster binding by the sdAbs. Similarly, the difference in K_D between sdAb 1 and 2 is entirely attributed to the K_{off} values, as the K_{on} values are identical. sdAb1 shows the slowest release of the molecules tested. This real-time binding information gives more insight into mechanistic aspects of binding than simply comparing K_D values.

Here, we explain how you can use FO-SPR to set up and run an assay that provides this advanced kinetic characterization. Generic carboxyl probes were used to screen the binding capacity of two different small molecules towards a biomarker. A set of different and distinguishable concentrations produced robust data, as indicated by the low 'parameters of error' Chi^2 and U-value. Moreover, in the case of the sdAb, two independent experiments were performed which obtained very similar results, indicating good reproducibility.

In conclusion, we demonstrate here that WHITE FOx is a powerful and an easy-to-use tool able to provide robust and reliable kinetic data.

Acknowledgement

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Related white papers

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White paper 2: Sensitive protein quantification in serum – comparison of FO-SPR and ELISA

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