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WHITE PAPER

Using FO-SPR to select for nanobodies in phage display

White paper 6 | Version 2 | Kris Ver Donck, Dagmara Minczakiewicz, Filip Delport, Kim Stevens

Abstract

Phage display in conjunction with biopanning is a frequently used strategy in the selection process for nanobodies and other expressed binding proteins with specificity to a target antigen. Phage are engineered to display a peptide of interest and be captured by conjugation to the desired target immobilized on a surface. Non-specifically bound phage are washed off and the bound phage are eluted to obtain the desired high-affinity clones which can then be used to create further display libraries.

Here we present an FO-SPR based approach that combines both fast kinetic characterization of phage binding and an efficient phage selection cycle in a single step. This technique presents a potential for phage selection based on the binding of molecules and interaction characterization, as it allows for real-time kinetics monitoring rather than the end point data from methods such as ELISA. Even avidity versus affinity binding can be assessed based on k_{on} and k_{off} behavior. Furthermore, it also avoids the risk of readout bias from affecting the binding of the secondary generic phage sandwich antibody needed in ELISA procedures.

The FO-SPR probes of the White FOx are ideally suited for this procedure. The user-friendly dip-in, fluidics-free setup offers a simple protocol for the capture, washing, and elution steps, while the binding affinity, association, and dissociation kinetics of the phage can be measured in real time.

Introduction

Phage display is a widely used method for generating screening libraries for peptides, nanobodies, and engineered antibodies to select for optimal target binding characteristics in clone screening and selection rounds. Common screening protocols make use of ELISA to identify strong binders, but this method is time consuming and only provides endpoint data, while kinetic data are the final goal. Furthermore, as many constructs are expressed with differing densities on the phage surface, and ELISA is typically performed with long incubation times, ELISA tends to show a bias towards slow binders and gives no information on avidity. Development of an integrated, one-step biopanning selection process with fast measurement provides a more convenient approach to overcome these drawbacks, by increasing efficiency in workflow execution and providing direct in-line monitoring of the key factor in the selection process: real-time kinetic binding and dissociation curves.

Viral expression systems such as M13 phage can be used to study the influence of copy number by expressing fusion peptides or proteins to any of their five coat proteins. Commonly used are the minor coat protein p3, with only five copies at the phage distal end, and the p8 protein, with up to 2,700 copies along the length of the viral filament. Furthermore, since phage are easily produced and the peptides they display retain their original conformation from the selection phase, those phage can be used directly as a scaffold for the peptide affinity labels.

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 analysis.

The SPR effect is achieved by coupling a white light source to the fiber optic sensor probe. In this consumable 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 1. The sensor probe is simply dipped into the liquid sample to measure biomolecular interactions directly. Figure 2 shows a schematic representation of how FO-SPR can be used for biopanning and Figure 3 shows a typical sensorgram resulting from a complete assay procedure across multiple phage titers as described in this white paper.

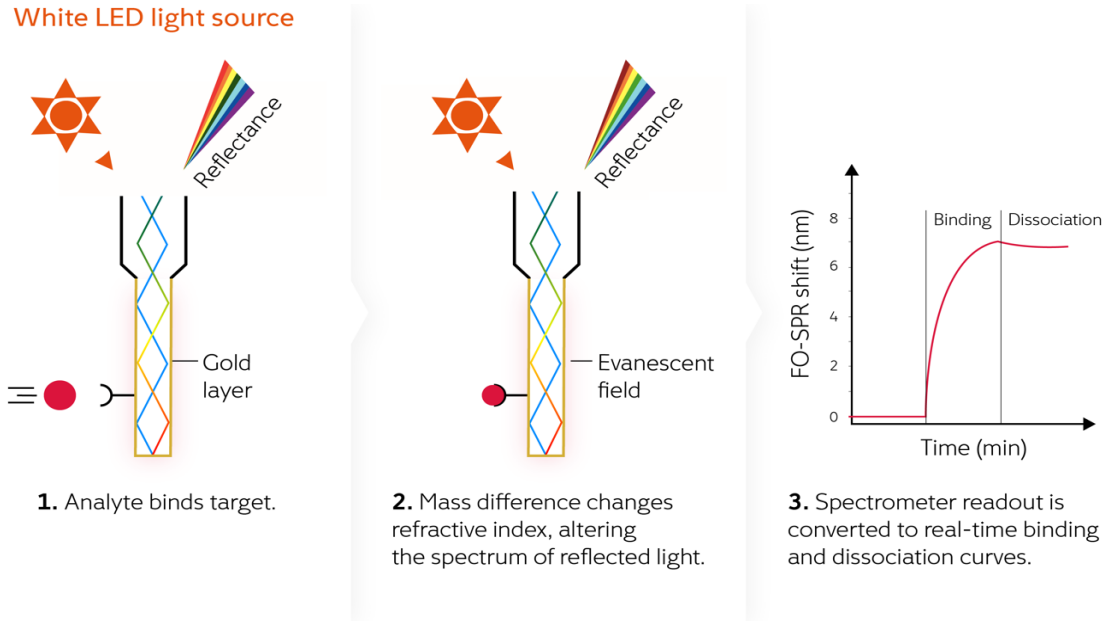


Figure 1: The FO-SPR principle.

Tools and reagents

To perform the procedures in this application you will need:

- White FOx instrument with FO-SPR acquisition software, the FOx data processing tool and kinetic analysis suite.
- Streptavidin sensor probes for label-free quantification with biotinylated capture molecules (FOx BIOSYSTEMS product nr: 30.0007) or carboxyl sensor probes for generic protein immobilization (FOx BIOSYSTEMS product nr: 30.0003).
- Microsoft Excel or other data calculation software for data handling and viewing.
- Micropipettes from 10 to 1000 µl with disposable tips.
- On-desk refrigeration for protein solutions.
- A microtube mixer and centrifuge.
- A phage display library expression and cloning platform, including bacteria growth media and shaking incubator. Here M13 phage were used as expression model1. Library generation and bacteria/phage culturing are not described in this white paper.

Reagents required for the 2 cases used in this white paper are listed in Table 1.

Buffer/Reagent	Concentration
Biotinylated eGFP	10 µg/mL
Lactoferrin	10 µg/mL in MEST
EDC: 1-ethyl-3-[3-dimethylaminopropyl] carbodiimide NHS: N-hydroxysuccinimide	0.4 M in EDC/NHS (see AN1) 0.1 M in EDC/NHS (see AN1)
PBST: Phosphate buffer saline, pH 7.4, 0.01% Tween 20	10 mM
MEST: 2-(N-morpholino) ethanesulfonic acid, pH 6.0 ,0.01% Tween-20	50 mM
PBS with 0.1% Tween 20, 1M NaCl	10 mM
Glycine buffers: Glycine (pH 2.5, pH 2.0, pH 1.5)	0.1 M

Table 1: Reagents and buffers required for FO-SPR. All solutions were prepared with deionized water purified by a Milli-Q Plus system.

This white paper makes use of either streptavidin prefunctionalized probes, which are ready-for-use to bind biotinylated capture protein in common lab buffers, or alternatively generic carboxyl probes. The latter can be used to immobilize a wide range of molecules and are not dependent on the availability of biotinylated or tagged materials, but they do require additional preparation as described in white paper 1: Carboxyl probe immobilization for label-free protein quantification. Both sets of probes are suitable for regeneration and in-run reuse, with the only limiting factors being the stability of the capture molecule and the strength of the target binding.

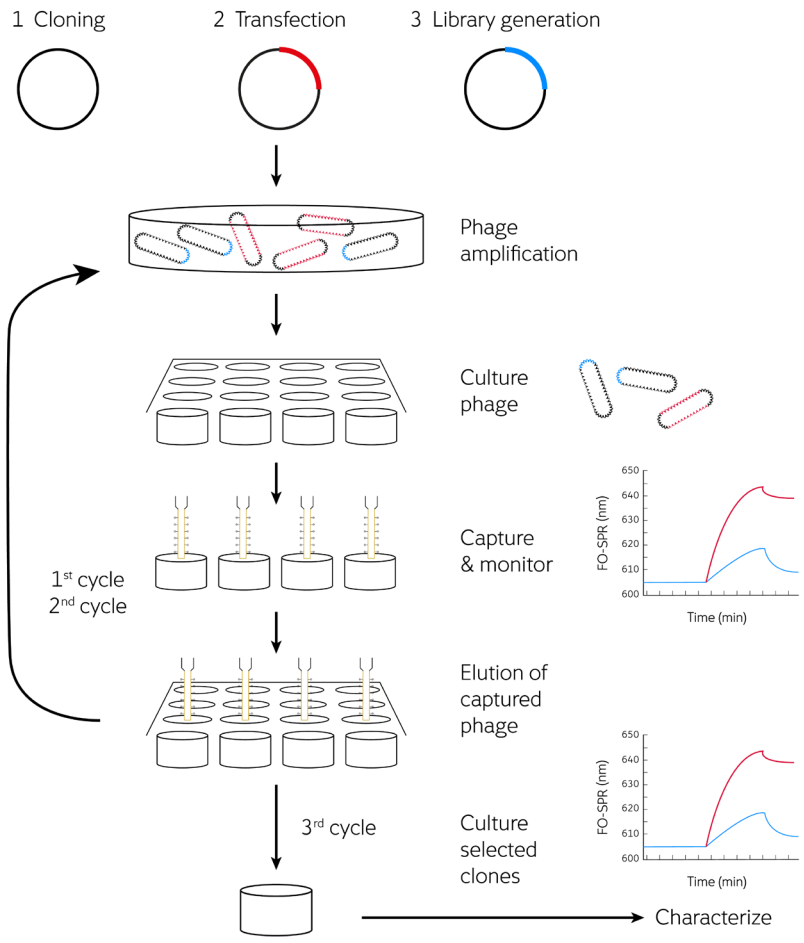


Figure 2: Schematic representation of the biopanning procedure using FO-SPR.

Biopanning to select for lactoferrin binding

As a model phage display system, libraries of M13 phage were used that displayed peptides with selective affinity for lactoferrin. The phage were incubated with *Escherichia coli* at 37 °C for 30 min. Infected cells were grown overnight in NZY-tetracycline-medium for phage amplification.

Carboxyl probes were functionalized with 10 µg/mL lactoferrin. Phage binding was performed for 10 minutes in MEST pH 6.0, and phage elution steps to collect cultures for the next growth cycle were performed for 5 minutes each in glycine buffers at pH 2.5, pH 2.0 and pH 1.5. Probes were regenerated for 60 s in PBS with 0.1% Tween 20, 1 M NaCl. An example protocol is shown in Table 2. All protocol steps can be performed at 26 °C, with either a low shaking speed of 200 rpm, or with shaking off (diffusion limiting).

Three rounds of selection were performed. From the third panning round, single clone phage were isolated, identified by DNA sequencing (data not shown), and tested for binding to lactoferrin using FO-SPR technology. Improved binding across 3 selection rounds is shown in Figure 5. Sensorgrams of selected clones with titrated amounts of viral load are shown in Figure 3.

Step #	Step name	Position	Time (s)	Step function
1	Probe pick-up	A1		Lactoferrin immobilized probes
2	Baseline	A1	600	Buffer equilibration
3	Association	A2	600	Target binding in phage suspension
4	Wash	A3	60	Wash off weakly bound phage
5	Elution	A4	300	Elution in Glycine pH 2.5
6	Elution	A5	300	Elution in Glycine pH 2.0
7	Elution	A6	300	Elution in Glycine pH 1.5
8	Regeneration	A7	60	PBST pH 6.0 1M NaCl
9	Probe drop off / or repeat step 4 in new position			End of cycle

Table 2: Programmed run sequence in White FOx software for the biopanning procedure using lactoferrin as a capture molecule. All steps are run with the shaker 'on' at minimum 200 rpm and temperature control set to 26 °C. The cycle time is 38 minutes.

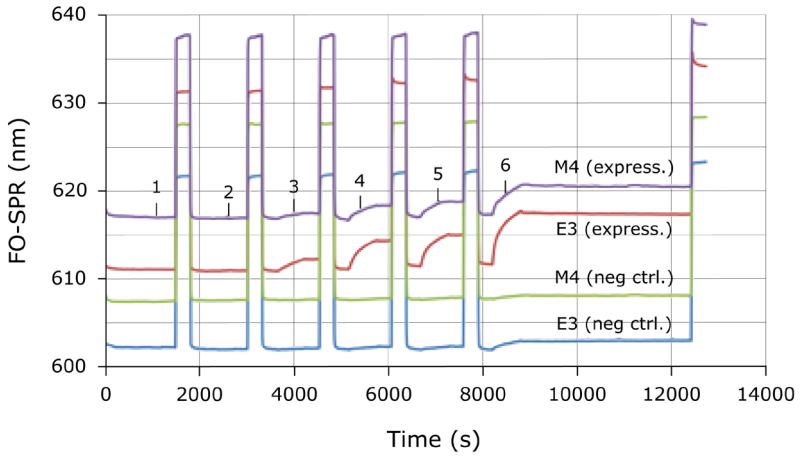


Figure 3: An example FO-SPR sensorgram showing binding of increasing concentrations of phage: (1) 0/mL, (2) $1x10^{10}$/mL, (3) $1x10^{11}$/mL, (4) $5x10^{11}$/mL, (5) $1x10^{12}$/mL, (6) $5x10^{12}$/mL using the label-free bioassay approach as described in the run sequence of Table 2. Results are shown for two different clones and their respective negative controls.

Kinetic analysis of clones selected by biopanning

As a model phage display system for characterizing binding kinetics, libraries of M13 phage were used that were displaying peptides with selective affinity for enhanced Green Fluorescent Protein (eGFP) either at the N-terminus of the p3 coat protein (end of filament, low number of binding sites) or p8 coat protein (side of filament, high number of binding sites) as illustrated in Figure 4. The phage were incubated with *Escherichia coli* at 37 °C for 30 min. Infected cells were grown overnight in NZY-tetracycline-medium for phage amplification. Three cycles of biopanning were performed, and the best-performing clones were characterized for binding kinetics.

As a simplified and faster workflow, 10 µg/mL biotinylated eGFP was used to functionalize the streptavidin coated fiber-optic sensor probe and to capture phage expressing eGFP binding peptides. Phage binding (at pH 6.0) and phage dissociation steps were performed for 15 minutes each.

Probes were regenerated for 60 s in PBS with 0.1% Tween 20, 1 M NaCl. This buffer acts as a mild regeneration buffer, not affecting the stability of the biotinylated eGFP. Again, the protocol can be performed at 26 °C, with either a low shaking speed of 200 rpm, or with shaking off (diffusion limiting).

After selection, the clones were analyzed to assess their binding characteristics with FO-SPR according to the protocol in Table 3 and compared to ELISA. Binding data are shown in Figure 5.

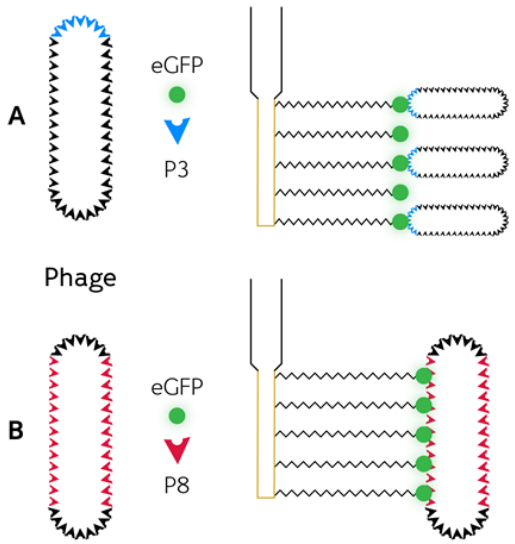


Figure 4: Schematic representation of the p3- and p8- transfected phage particles, showing the different numbers of eGFP binding sites per phage.

Step #	Step name	Position	Time (s)	Step function
1	Probe pick-up	A1		Probe signal reference
2	10mM MEST pH 6.0	A1	600	Probe wetting
3	eGFP	A2	900	Biotin-streptavidin binding
4	Baseline (MEST)	A3	300	Buffer wash (remove non-bound eGFP)
5	Association (phage in MEST)	A4	900	Target binding in phage suspension
6	Dissociation (MEST)	A5	900	Dissociation in suspension buffer
7	Regenerate (BPST)	A6	60	Wash off bound phage
8	Probe drop off / or repeat step 5 in new position			End of cycle

Table 3: Programmed run sequence in White FOx software for binding curve characterization. All steps are run with shaker 'on' at 200 rpm and temperature control set to 26 °C. The cycle time including regeneration is 36 minutes.

Results

Biopanning

While the first biopanning selection of lactoferrin-binding phage only gave a low response on FO-SPR (Figure 5), the subsequent second and third rounds showed clear enrichment with more strongly-binding phage.

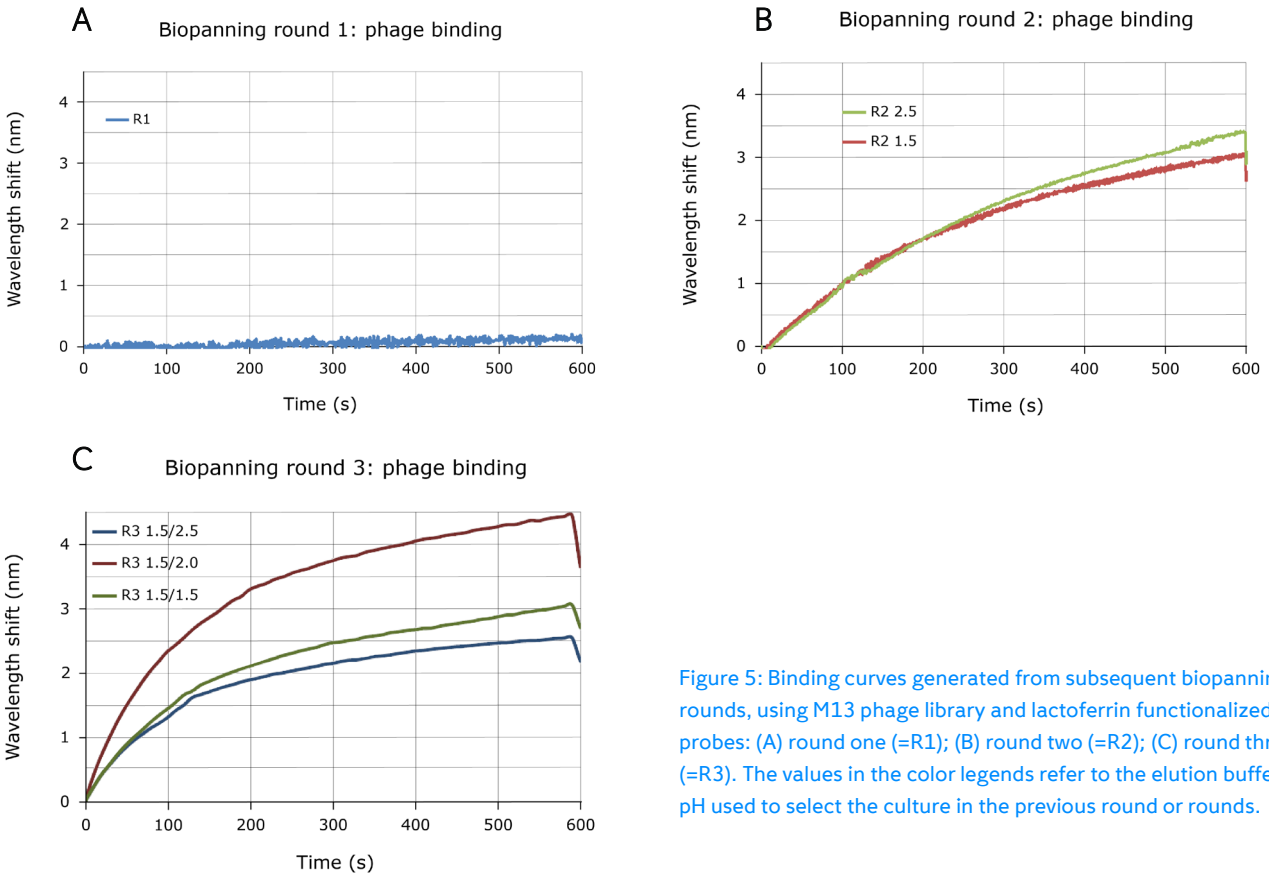


Figure 5: Binding curves generated from subsequent biopanning rounds, using M13 phage library and lactoferrin functionalized probes: (A) round one (=R1); (B) round two (=R2); (C) round three (=R3). The values in the color legends refer to the elution buffer pH used to select the culture in the previous round or rounds.

Kinetic characterization of clones selected by biopanning

In a separate experiment, 3 rounds of biopanning with phage expressing eGFP binding peptides were performed. Binding characterization of the eGFP phage was conducted at $2x10^{12}$ phage/mL. Although all p3 phage clearly showed different interactions with eGFP compared to the native phage, significantly higher SPR signal shifts were noted for the p3 phage expressing a short repeat sequence of binding peptide. p8 phage, with a large number of binding sites (Figure 4), showed a higher affinity for eGFP than all p3 phage. Avidity is expected to increase from p3 with single peptides, to p3 with peptide repeats, to p8 phage with more copies of single peptides, shown in Figure 6 as a general rise in signals. The kinetic information of k_{off} and k_{on} allows the ranking of affinity performance over avidity performance spanning the different phage types.

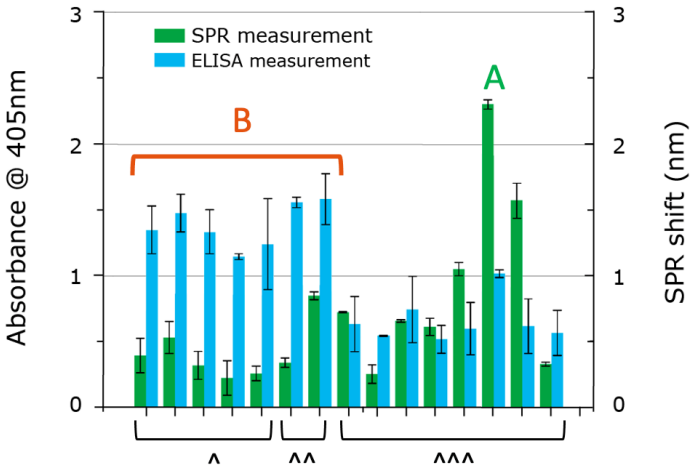


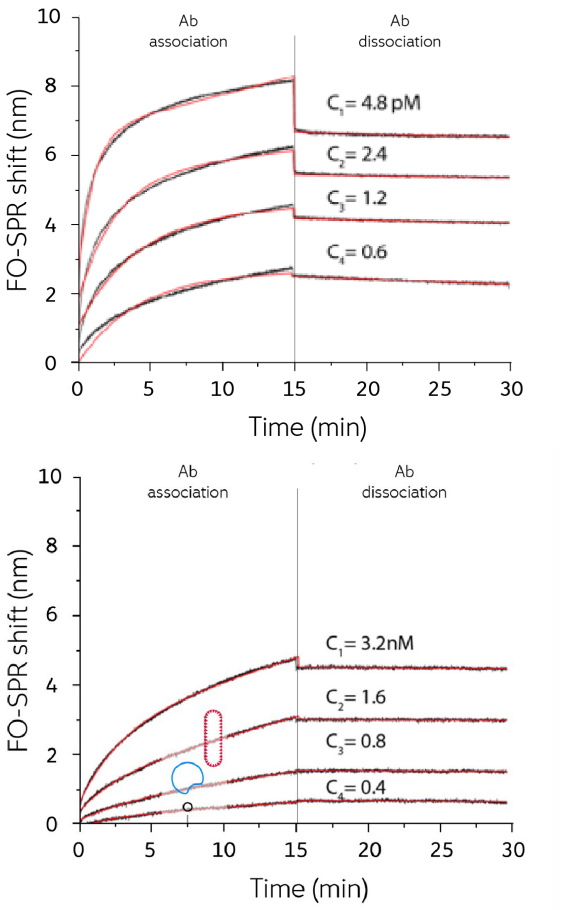
Figure 6: Comparison of FO-SPR vs ELISA for M13 eGFP-binding peptide expressing clones with a low number of P3-expressed binding sites (^), elevated number of P3-expressed binding sites (^^), and high number of P8-expressed binding sites (^^^) to eGFP. The FO-SPR top scoring clone (A) scores lower on ELISA and the ELISA top scoring clones (B) score lower on FO-SPR. Reprinted (adapted) with permission from *Anal. Chem.* 2013, 85, 21, 10075–10082 https://doi.org/10.1021/ac402192k. Copyright 2013 American Chemical Society.[1]

Comparison with ELISA

SPR measurements on the eGFP-binding phage clones were further validated with an endpoint ELISA test (Figure 6). The ELISA resulted in an indistinguishable difference in signal between the 2 types of p3 phage, whereas FO-SPR was able to distinguish the signal increase of p3 expressing a short repeat sequence of binding peptide over the p3 phage expressing a single binding peptide. While p3 phage gave a much higher signal in the ELISA assay than p8 phage, the SPR binding signals showed the reverse trend. This may be due to the sandwich assay making use of an anti-p8 secondary antibody, the binding site of which can be affected by the peptides expressed in the p8 protein

Kinetic FO-SPR analysis

For a set of selected phage clones, the kinetic binding parameters (k_{on}, k_{off} and K_d) were derived from the individual SPR measurements. Figure 7 shows that the top scoring clone on FO-SPR showed a K_d of $1.2\pm0.1x10^{-14}$ M, compared to a K_d of between $1.1x10^{-11}$ M and $4.5x10^{-12}$ M for the other clones. All clones had a k_{off} between 1.0 and $6.0x10^{-3}$(1/s). Although avidity is present, the results show that its effect could likely be attributed to the k_{on}, since the top clone from FO-SPR resulted in $1.9x10^{11}$ (1/sM) while the other clones all scored in the 10^8(1/sM) range.



Conclusions

This study demonstrates the FO-SPR based kinetic analysis of protein binding affinities during a biopanning procedure. Whole phage particles were used that expressed peptides selective for lactoferrin binding. The main advantages of this method are the combination of fast panning selection cycles of only half an hour and the immediate readout of the crude sample's binding profile. No protein isolation or sample purification was required.

Moreover, the same FO-SPR method can provide advanced kinetic characterization, without further assay optimization being necessary. Its use for kinetic characterization was demonstrated in an experiment using M13 phage expressing peptides for eGFP binding on either the minor (p3) or major (p8) coat proteins. The label-free kinetic characterization allows the individual determination of k_{on} and k_{off}, in addition to K_d. This makes it possible to determine the contribution of both the on and off rates to the K_d for each clone, which is not possible with an endpoint method like ELISA. With a short time to result and no risk of bias from the interactions with secondary antibodies in a sandwich assay, this FO-SPR method gives insights into affinity and avidity and allows a more detailed selection of strong versus weak binders, as well as fast versus slow binders.

Feature	FO-SPR	ELISA
k_{on}	yes	no
k_{off}	yes	no
K_d	yes	yes (static)
Crude samples	yes	limited
Label free	yes	no
Short time to result	yes	no

Table 4: A summary of the capabilities of FO-SPR and ELISA.

Figure 7: Kinetic profiles of the FO-SPR top scoring clone (top) with a K_d of $1.2x10^{-14}$ M while scoring lower on ELISA and one of the ELISA top scoring clones (bottom) with a K_d of $1.1x10^{-11}$ M while scoring low on FO-SPR. Reprinted (adapted) with permission from *Anal. Chem.* 2013, 85, 21, 10075–10082 https://doi.org/10.1021/ac402192k. Copyright 2013 American Chemical Society.[1]

Acknowledgement

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References

1. Knez et al., Affinity Comparison of p3 and p8 Peptide Displaying Bacteriophages Using Surface Plasmon Resonance (2013) Anal. Chem. 85, 10075−10082

Related white papers

White paper 1: Carboxyl probe immobilization for label-free protein quantification, describes how to approach optimization of immobilization to the carboxyl FO-SPR probes.

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