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APPLICATION NOTE

SENSITIVE PROTEIN QUANTIFICATION IN CRUDE SERUM SAMPLES: COMPARISON OF FO-SPR AND ELISA

Application note 2 | Kim Stevens, Kris Ver Donck, Filip Delport

Based on original publication: Lu et al. (2016) Biosensors and Bioelectronics 79, 173–179. (1)

Abstract

This application note demonstrates the potential of FOx BIOSYSTEMS' fiber-optic surface plasmon resonance technology (FO-SPR) for determining the concentration of a protein or antibody in crude samples, like serum. As an example, we demonstrate the detection of the therapeutic monoclonal antibody, infliximab (IFX), in patient serum samples. FO-SPR is a powerful tool that harnesses the power of SPR in an easy-to-use fiber-optic sensor.

The methods described here demonstrate that FO-SPR, enhanced with a gold nanoparticle sandwich-style bioassay, can detect biologically relevant concentrations of infliximab. IFX levels in patient serum are typically between 0.5 – 10 µg/ml, and the lower detection limit of this assay was 0.25 ng/ml or 1.7pM in 100-fold diluted patient serum. Furthermore, there is an excellent correlation between results obtained by FO-SPR and those using an optimized ELISA technique (Pearson correlation 0.998 and intraclass coefficient 0.982).

We discuss how the FO-SPR sandwich-style assay can provide a fast and accurate alternative to ELISA for the analysis of biomolecules in crude samples with minimal processing, thereby showing great potential as an R&D tool.

Introduction

Crude biological samples often present challenges when quantifying biomolecules of interest during the research and development of diagnostics or therapeutics. To overcome these challenges, purification steps may be used, but these can introduce a bias in the experimental results, as well as more hands-on time and cost. Therefore, a direct method of detection often is preferred.

Here, we use infliximab (IFX) to demonstrate direct detection in serum samples. IFX is a therapeutic monoclonal antibody used in the treatment of inflammatory bowel disease. If the dose is too low or too high, many patients lose responsiveness to IFX over time, requiring dose or therapy adaptations to maintain effectiveness. The optimal dose depends on the therapeutic drug level in the patients' serum just prior the administration. Therefore, fast and sensitive measurement in patient samples is crucial for timely dose adaptation to optimize therapeutic outcomes and minimize costs.

The most commonly used detection method is enzyme-linked immunosorbent assay (ELISA); however, this is a slow technique, taking around 1.5 days. A faster 2-hour ELISA test has been developed for infliximab detection, but this still requires a well-equipped lab and falls outside of the patient appointment time window.

Surface plasmon resonance (SPR) is becoming increasingly used as an alternative biosensing technology and offers a real-time alternative to ELISA that can be used with and without labels. SPR exploits electromagnetic waves to measure local refractive index changes due to suspended analytes and immobilized biomolecules on the sensor surface. The most commonly used SPR biosensors are sensitive, reliable and accurate, however, they typically use microfluidics and an optical path with prisms coated with a thin gold layer and are often unsuitable for quick testing due to their size and cost.

Fiber-optic surface plasmon resonance (FO-SPR) offers a convenient alternative to both conventional SPR biosensors and ELISA. Here, the gold layer is applied to fiber optic SPR probes which can be dipped into the sample of interest. This approach capitalizes on the speed and performance of SPR in an easy-to-use dip-in protocol which eliminates the contamination issues often found when analyzing serum with microfluidics instruments. FO-SPR has been incorporated into a benchtop instrument: the White FOx by FOx BIOSYSTEMS.

This application note compares two different techniques for the quantification of infliximab in the serum of patients:

ELISA and FO-SPR. IFX levels in patient serum are typically between 0.5 – 10 µg/ml. Therefore, in order to enhance the SPR signal to a relevant detection range matching the dilution of sample in the ELISA, a sandwich assay format was used here, with gold nanoparticles bound to detection antibody. FO-SPR is ideally suited to this application, due to its speed, sensitivity, and ease of use. Its fluidics-free protocol requires minimal processing of the patient serum. For even faster analysis of proteins in whole blood with minimal processing, please see application note 3: Sensitive protein quantification in blood, dried blood spots, serum and plasma.

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 end 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 typical sensorgram resulting from the full procedure antibody immobilization and IFX detection procedure described in this application note.

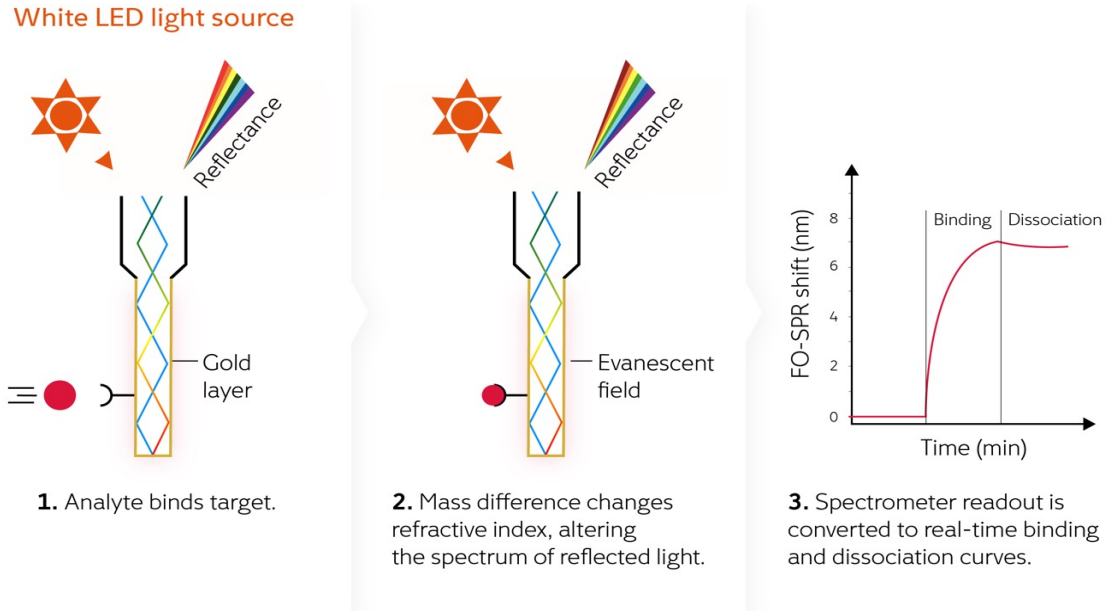

Figure 1: FO-SPR principle

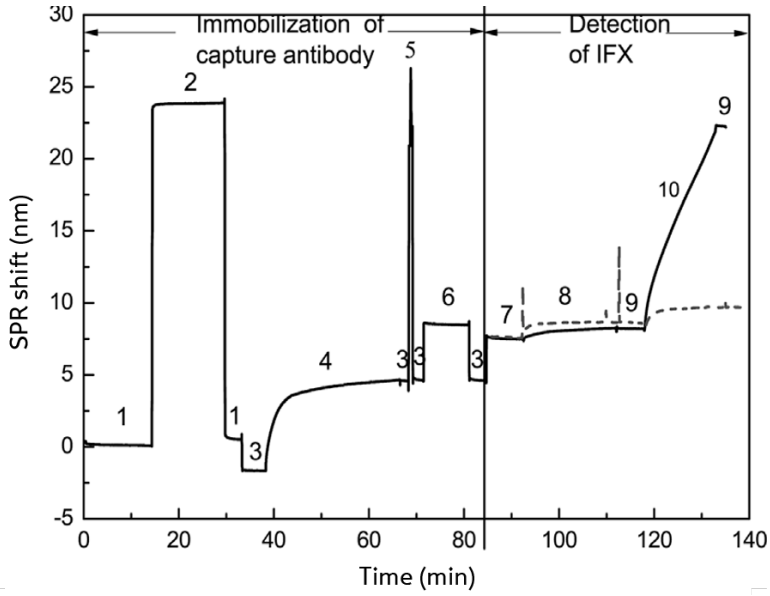

Figure 2: A typical FO-SPR sensorgram showing all steps of covalent immobilization of antibodies and IFX detection using the sandwich bioassay approach. The dashed line on the detection part represents the detection of 10 µg/ml of IFX using signal amplification with 20 µg/ml of MA-IFX6B7 without AuNPs.

1. MES buffer priming
2. EDC/NHS surface activation
3. Sodium acetate buffer pH 5.5
4. Capture antibody immobilization
5. Regeneration buffer: 50 mM NaOH / 1 M NaCl
6. Blocking buffer: 50 mM ethanolamine in PBS
7. PBS with 0.01% Tween 20
8. IFX binding
9. PBS with 0.5% BSA
10. AuNPs sandwich amplification

Tools and reagents

To perform the procedures in this application you will need:

- White FOx instrument with FOx-SPR acquisition software and the FOx data processing tool
- Carboxyl probes for label-free quantification (FOx BIOSYSTEMS product nr: 30.0003)
- Microsoft Excel or other data calculation software for data handling and viewing
- Micro pipettes from 10 to 1000 µl with disposable tips
- On-desk refrigeration for protein solutions
- Vertically rotating shaker
- Costar high binding 96-well plates (ELISA)
- Shaker (ELISA)
- ELx808 Absorbance Microplate Reader (ELISA)

Buffer/Reagent	Concentration
MES: 2-(N-morpholino) ethanesulfonic acid, pH 6.0	50 mM
EDC: 1-ethyl-3-[3-dimethylaminopropyl] carbodiimide	0.4 M in EDC/NHS
NHS: N-hydroxysuccinimide	0.1 M in EDC/NHS
Anti-IFX-C monoclonal capture antibody	1 mg/ml
NaOH: Sodium hydroxide	50 mM in NaOH/NaCl
NaCl: Sodium chloride	1 m in NaOH/NaCl
PBS: Phosphate buffer saline, pH 7.4	10 mM
Ethanolamine (in PBS)	50 mM
Tris (in PBS)	50 mM
Blocking solution (Dojindo laboratories)	
Anti-IFX-D monoclonal detection antibody	1 to 2.5 mg/ml stock 20ml, optical density (OD) 1
20nm diameter gold nanoparticles EMGC20	Average size 19.7nm (CV 8%)
Sodium carbonate	0.2 mM
Bovine serum albumin (BSA)	0.5% w/v
BSA in PBS	0.5% and 1%
Sodium acetate buffer, pH 5.5	10mM
infliximab (Remicades)	Received lyophilized Resuspended to 1 mg/ml stock
Tween-20	0.01% in PBS
Pooled serum from 30 healthy volunteers	(1)
De-identified serum samples from five IFX-treated IBD patients	(1)
TNF (ELISA)	240 ng/ml (3)
pAB-IFX-HRP or HRP-conjugated MA-IFX (ELISA)	20 mg/ml (3)
o-phenylenediamine and H_2O_2 in citrate buffer (ELISA)	400 µg/ml - $3x10^{-3}$% (3)
H_2SO_4: Sulfuric acid (ELISA)	2 M (3)

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

Note: Special attention is required for EDC/NHS which is a highly reactive and short-lived reagent (EDC user guide, Sigma Aldrich) and should be prepared just prior to use. Separately dissolve NHS and EDC no more than 5 minutes before use, mix in equal quantities, and pipet into the designated wells immediately before use.

Alternatively, you can also prepare pre-dissolved aliquots of EDC and of NHS, respectively, and store them at -20°C until use as explained in the application note nr 1 about immobilization on carboxyl probes. Thaw one aliquot of each at room temperature for about 20 minutes before use and mix in the designated wells just before placing the reagent in the instrument.

Surface functionalization of FO probes with capture antibody

Carboxyl probes were primed 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 15 minutes. A 20 µg/ml solution of anti-IFX-C monoclonal capture antibody in a 10mM sodium acetate buffer at pH 5.5 was covalently immobilized to the activated carboxyl groups for 30 minutes. Two 30-second washes in regeneration buffer (50 mM NaOH/1 M NaCl) removed non-covalently bound antibodies. Three different blocking buffers were tested (50 mM Tris in PBS, 50 mM ethanolamine in PBS and commercial blocking solution from Dojindo Laboratories) by immersing the probes for 8 minutes to deactivate unreacted carboxylic groups.

Surface functionalization of gold nanoparticles with detection antibody

adsorption using a modified protocol based on that from Jans *et al.* (2). The AuNP solution was adjusted to pH 9.2 using 0.2 mM sodium carbonate prior to use. Antibody solution was added to 800 µL of 7 x 1011 particles/ml AuNP solution to give a final antibody concentration of 5 µg/ml and incubated at room temperature on a vertically rotating shaker for 20 min. 560 µL of bovine serum albumin (BSA) (0.5% w/v) was added to stabilize the particles and the mixture was returned to the shaker for 1 hour at room temperature. The solution was then centrifuged at 7000 rpm for 30 min at 20°C. After discarding the supernatant containing unbound antibodies, the nanoparticle residue was resuspended in PBS with 0.5% BSA. In order to ensure reproducibility, the final concentration of AuNPs was adjusted to be the same as pre-functionalized AuNPs (7x1011 particles/ml, OD of 1). The prepared AuNPs were stored at 4°C until use. 150 µL of functionalized AuNPs were used in each assay.

Non-specific binding controls

Non-specific binding between the various assay components was tested under a variety of scenarios:

1. Between IFX target molecules and the sensor surface without capture antibody, after deactivation with blocking buffers (50 mM Tris in PBS, 50 mM ethanolamine in PBS and commercial blocking solution from Dojindo Laboratories).
2. Between sample matrix components and immobilized capture antibody using the three different blocking buffers mentioned above.
3. Between AuNPs functionalized with detection antibodies and the capture antibody immobilized on the sensor surface in 100-fold diluted serum.
4. Between monoclonal capture and detection antibodies with ADM, another anti-TNF biological.

Establishing the FO-SPR assay for IFX detection in serum

FO probes functionalized with anti-IFX-C capture antibodies were used to detect 10 µg/ml or 1 µg/ml IFX diluted in PBS with 0.01% Tween 20 for 20 minutes to match the ELISA conditions.

Signal amplification was obtained using the detection antibodies in a sandwich assay format to detect IFX in serum spiked with an IFX concentration series (0, 2.5, 5, 10, 20, 25, 40, 50, 75 ng/ml) diluted with PBS / 0.01% Tween 20.

The functionalized FO probes were immersed in the IFX dilution for 15 minutes, followed by 20 minutes of signal amplification using functionalized AuNPs. The probe surfaces were washed with regeneration buffer (50 mM NaOH / 1 M NaCl) prior to each IFX concentration for 2 x 30 seconds to remove any bound material.

IFX quantification in patient sera using FO-SPR

Serum was tested from five IFX-treated patients in quadruplicate using the methods described in the above section. FO probes were functionalized with capture antibody and a bioassay format with AuNPs conjugated with detection antibody (anti IFX-D) was applied as described in the above section. Each sample was diluted 200-fold and measured 4 times using 2 independently prepared batches of fibers and AuNPs to estimate inter-assay variation.

IFX quantification in patient sera using ELISA

Each sample was also tested by ELISA using previously optimized conditions for IFX detection (3). Each sample was measured three times on different days. 96-well plates were coated overnight with TNF at 4°C, then blocked with PBS / 1% BSA for 2 hours at room temperature. Samples were diluted 1:150 and 1:300 in PBS / 1% BSA, added to the plate, and incubated for 2 hours at 37°C on a shaker. Plates were then washed and pAb-IFX-HRP or HRP-conjugated MA-IFX was applied for detection of bound IFX and incubated for 1 hour at room temperature. Plates were washed and developed using o-phenylenediamine and H2O2 in citrate buffer, and the reaction was stopped with 2 M H2SO4. The absorbance was measured at 490 nm with an ELx808 Absorbance Microplate Reader.

The assay cut-off was 0.2 µg/ml of IFX and lower limit of quantification was 0.5 µg/ml of IFX for 150-fold diluted serum.

Results

Non-specific binding controls

IFX only binds specifically to the biosensor in the presence capture antibody. The interaction between sample matrix components and immobilized capture antibody was low. T capture and detection antibodies have a high specificity against IFX and not against other antibodies or anti-TNF biologics. For serum spiked with 10 µg/ml IFX, the largest signal-to-noise ratio and smallest inter-experiment variab was achieved using ethanolamine in PBS. This was therefc used as blocking buffer in all subsequent experiments. For results see the original paper by Lu et al (4).

Calibration curve generation

In order to quantify IFX in patient serum samples, calibrati curves were generated for IFX spiked in both buffer and se where the SPR shifts were plotted against a function of concentration as shown in figure 3.

The lower limit of detection was calculated to be 2.2 ng/m (15 pM) in 100-fold diluted serum from healthy volunteers.

The concentrations of unknown samples were checked by interpolating the SPR signal into the calibration curve using anti IFX-D AuNPs and less than 5% difference was observed between known and determined concentrations from interpolation with the calibration curve.

IFX measurement in IFX-treated patient sera

The IFX concentrations for sample S2 were indistinguishable from healthy serum using both FO-SPR and ELISA. This was corroborated after deblinding the samples, when it was found that patient S2 was naïve to IFX therapy. The IFX concentration for sample S3 was beyond the dynamic range of the FO-SPR calibration curve when diluted 200-fold, therefore it was diluted 500-fold and interpolated on the original 200-fold calibration curve.
The coefficient of variation between the four measurements of each individual sample by FO-SPR was less than 10% and the inter-assay coefficient of variation was less than 10% for both FO-SPR and ELISA.
There was excellent correlation between IFX concentrations measured with FO-SPR and ELISA as shown in figure 4 (Pearson correlation 0.998 and intraclass coefficient 0.983).

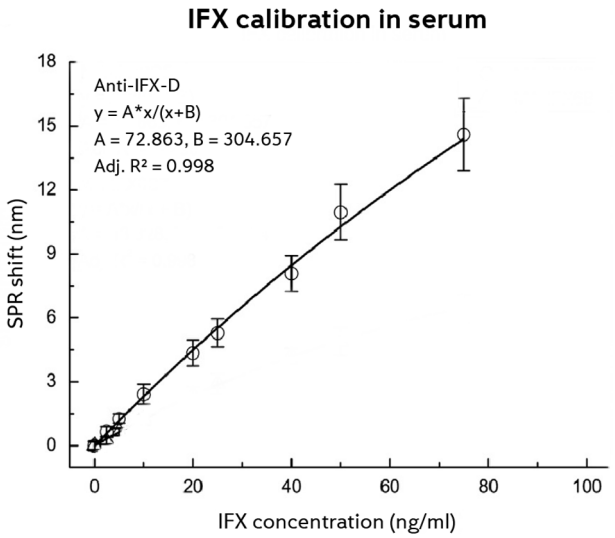

Figure 3: Calibration curve for spiked IFX in serum.

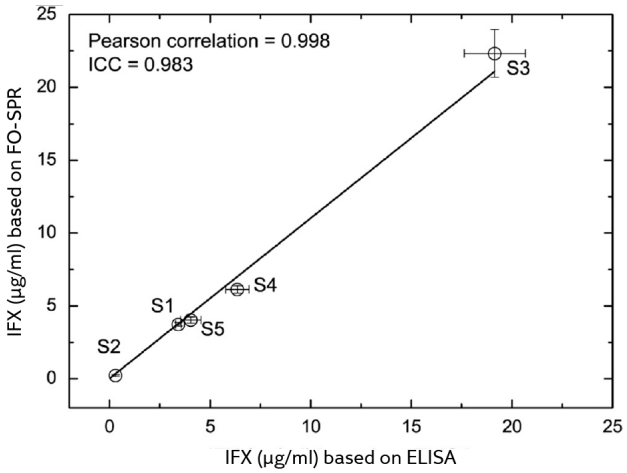

Figure 4: Evaluation of the FO-SPR assay with serum samples from IBD patients compared to the results from an in-house developed IFX-specific ELISA test. Pearson correlation coefficient: 0.998 and intraclass coefficient: 0.983. Error bars represent standard errors (n=4 for FO-SPR and n=3 for ELISA).

Discussion / Conclusion

When measuring proteins or antibodies in research samples, it is essential for tests to be sufficiently sensitive to detect and quantify at biologically relevant concentrations. In addition, the ability to deal with complex samples, such as serum, without the need for time-consuming purification procedures to avoid cross-contamination or clogging in microfluidics systems is highly advantageous.

Thanks to the unique FO-SPR probe setup, the SPR signal can easily be amplified using a sandwich assay format with gold nanoparticles conjugated to detection antibody. IFX concentrations in patient serum samples are typically between 0.5 – 10 µg/ml, therefore with a lower limit of detection of 2.2 ng/ml (15pM) in 100x diluted serum, FO-SPR AuNP sandwich assays shows great potential for this application.

The results obtained by FO-SPR showed excellent correlation with those obtained from an IFX-optimized ELISA test, the current gold standard for IFX quantification. The lower limits of detection were also similar to the 0.5 µg/ml calculated from 150-fold diluted serum obtained using ELISA.

As presented here, the total assay time of 2.5 hours including capture antibody immobilization is already substantially faster than the ELISA test which has an overnight incubation. With a prefunctionalized probe, the assay time can be reduced to under 20 minutes. Together with kinetic analysis, this would even bring the assay time under 10 minutes.

This study demonstrates FO-SPR to be a user-friendly technology which combines the speed and accuracy of SPR with the advantage of fluidics-free dip-in sensing. It has potential applications in antibody and protein production, and even microvesicle or phage particle quantification.

Furthermore, since FO-SPR is fully automated and can either run individual measurements or with 4 parallel FO sensors, it shows great potential to determine biological drugs in patient serum samples both for a limited number of samples as well as in high throughput mode.

For an even faster method of protein quantification in blood, as well as serum and plasma, please see application note 3: Sensitive protein quantification in blood, dried blood spots, serum and plasma.

Acknowledgement

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References

1. Lu et al. (2016) Fiber optic-SPR platform for fast and sensitive infliximab detection in serum of inflammatory bowel disease patients. Biosensors and Bioelectronics 79, 173–179.

2. Jans et al. (2009) Dynamic light scattering as a powerful tool for gold nanoparticle bioconjugation and biomolecular binding studies. Anal. Chem. 2009, 81, 9425–9432.

3. Van Stappen et al. (2015) Generation of a highly specific monoclonal anti-infliximab antibody for harmonization of TNF-coated infliximab assays. Ther. Drug Monit. 37 (4), 479–485.

Related application notes

How to optimize immobilization to the FO-SPR probes is described in application note 1: Carboxyl probe immobilization for label-free protein quantification.

A faster FO-SPR protocol for quantifying proteins in complex matrices, including whole blood, is described in application note 3: Sensitive protein quantification in blood, dried blood spots, serum and plasma.

How to set up a label-free quantification assay starting from an immobilized capture surface is described in application note 4: Label-free protein quantification.

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