



Fiber optic-SPR platform for fast and sensitive infliximab detection in serum of inflammatory bowel disease patients



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ABSTRACT

Infliximab (IFX) is a therapeutic monoclonal antibody used for treating patients with inflammatory bowel disease (IBD). In order to improve therapeutic outcomes it is recommended to monitor IFX trough concentrations. Although ELISA is currently widely used for this purpose, this method is not suitable for single patient testing. In this paper we describe the development of a fast bioassay for determining IFX concentration in serum using an in-house developed fiber-optic surface plasmon resonance (FO-SPR) biosensor. Studies were first conducted to optimize covalent immobilization of the IFX-specific antibody on the sensor surface as well as to select an optimal blocking buffer for restraining the non-specific binding. In order to reach clinically relevant sensitivity for detecting IFX in patients' serum, the SPR signal was amplified by employing gold nanoparticles functionalized with another set of IFX specific antibodies. Using the optimized sandwich bioassay, calibration curves were made with series of IFX concentrations spiked in buffer and 100-fold diluted serum, reaching the limit of detection of 0.3 and 2.2 ng/ml, respectively. The established bioassay was finally validated using five IFX treated IBD patients samples. Results from the FO-SPR platform were compared with an in-house developed, clinically validated ELISA resulting in excellent Pearson and intraclass correlation coefficient of 0.998 and 0.983, respectively. Furthermore, the assay time of the FO-SPR platform was significantly reduced compared to ELISA, demonstrating the potential of this platform to be used as a point-of-care diagnostic tool for improving therapeutic outcomes of IBD patients.

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1. Introduction

Inflammatory bowel disease (IBD) is a chronic, relapsing inflammation of the gastrointestinal tract, which limits the life quality of patients affected by the disease. Although incurable, the symptoms can be controlled by medications such as steroids, aminosaliclates, immunomodulators and by using monoclonal antibodies, which target tumor necrosis factor alpha (TNF- α). Infliximab (IFX), a therapeutic monoclonal antibody specifically targeting TNF- α , has been proven as highly effective for inducing and maintaining remission in moderate-to-severe IBD patients. Although the therapy with biologics has revolutionized the management of IBD patients compared to conventional treatments, the average annual cost of IFX per patient is rather high, between \$24000 and \$26000 (approximately €18,700–20,000) (Bonafede

et al., 2012). Typically, an IFX infusion dose of 5 mg/kg is administered at week 0, 2 and 6, followed by maintenance therapy at 8 week intervals (Sandborn and Hanauer, 2002). However, some patients, who initially respond to the induction therapy, lose the response over time, requiring therefore adjustment of dose and/or interval time. For improving the long-term therapeutic outcomes of IBD patients, it is recommended to monitor the IFX trough concentration, which is defined as the lowest drug concentration immediately before the next infusion is administered (Vermeire and Gils, 2013). During maintenance therapy, clinical response is associated with IFX trough concentrations in the range from 0.5 to 10 μ g/ml in serum (Steenholdt et al., 2011). The most commonly used detection method is the enzyme linked immunosorbent assay (ELISA). Although a faster ELISA test is already available on the market (apDia, Turnhout, Belgium), to replace previous ELISA tests taking 1.5 days (Ternant et al., 2006; Vande Castele et al., 2012), time to obtain results still requires at least 2 h and can only take place in well-equipped central laboratories, which hampers immediate dose adaption. Therefore, a faster detection system is

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highly desired as a point-of-care (POC) diagnostic tool that can replace ELISA without reduction in sensitivity or specificity (Van Stappen et al., 2015c).

Over the last three decades, optical biosensors based on surface plasmon resonance (SPR) have been increasingly employed in a variety of applications including food safety, clinical diagnostics and environmental monitoring (Pollet et al., 2011; Yanase et al., 2013; Zhang and Fang, 2010). SPR biosensors are optical sensors exploiting electromagnetic waves known as surface plasmon waves to measure local refractive index changes due to interactions between analytes in solution and biomolecules immobilized on the sensor surface. The most commonly used SPR biosensors, including commercially available Biacore instruments, exploit prisms coated with a thin gold layer using the Kretschmann configuration (Schasfoort and Tudos, 2008). Although prism-based SPR biosensors are sensitive, reliable and accurate, they are usually bulky and expensive and therefore are not suitable for POC diagnostics which have been highly demanded in recent years. Standard optical fibers have emerged over the years as excellent alternatives that can compete with the sensitivity and specificity of prism-based SPR sensors, while offering the advantage of low cost and small size greatly desired for developing a POC tool. One such platform, namely fiber optic surface plasmon resonance (FO-SPR), has been previously described by our group as a promising biosensor for protein- and DNA-based assays (Delpont et al., 2012; Janssen et al., 2012; Knez et al., 2013, 2014; Pollet et al., 2011, 2009; Tran et al., 2011). The FO-SPR biosensor makes use of multimode optical fibers that facilitate SPR generation and enable implementation of bioassays. Moreover, the FO-SPR platform offers several advantages compared to many current standard techniques, including real-time monitoring, fast response time and ease of operation. This, combined with proven high sensitivity and specificity in detecting both proteins and DNA molecules, reveals the vast potential of the FO-SPR platform in developing clinical diagnostic tool for applications such as monitoring IFX trough concentrations in IBD patients.

In this study, we present the development of a fast and sensitive FO-SPR-based immunoassay for determining IFX concentrations in serum samples from IBD patients. First, selection of buffers and range of antibody concentrations was studied for immobilizing in-house developed IFX specific antibodies on the sensor surface. Moreover, a blocking buffer was chosen to provide a maximal signal-to-noise ratio for IFX detection in serum. In order to reach clinically relevant sensitivity for detecting IFX in patients' serum, the SPR signal was amplified by employing gold nanoparticles (AuNPs) functionalized with another set of in-house developed IFX specific antibodies. Calibration curves were made with IFX spiked to buffer, 100-fold and 200-fold diluted serum. Finally, the performance of the developed FO-SPR bioassay was evaluated using five serum samples from IFX treated IBD patients and results were compared using an IFX ELISA (Van Stappen et al., 2015a). The obtained results combined with other intrinsic features of the FO-SPR platform, such as real-time monitoring, fast response time and ease of operation, demonstrate its huge potential for deployment as a POC diagnostic tool in determining biological drugs in patients' sera.

2. Materials and methods

2.1. Buffers and reagents

All buffer reagents were obtained from Sigma-Aldrich (Bornem, Belgium), unless stated otherwise. All solutions were prepared with deionized water purified by a Milli-Q Plus system (Millipore, Marlborough, MA, USA). Acetone, sulfuric acid (97% H₂SO₄) and

acetic acid were purchased from Chemlab (Zedelgem, Belgium). Tween 20 was provided by AppliChem GmbH (Darmstadt, Germany). Carboxylic acid-SAM formation reagent, biotin-SAM formation reagent and amine coupling kit were produced by Dojindo Laboratories (Kumamoto, Japan). 1-ethyl-3-[3-dimethylaminopropyl] carbodiimide (EDC) and N-hydroxysuccinimide (NHS) were obtained from Thermo Fisher Scientific (Erembodegem, Belgium). o-Phenylenediamine was purchased from Acros Organics (Geel, Belgium). AuNPs EMGC20 with the diameter of 20 nm were provided by BBI Solutions (Cardiff, UK). Information on the diameter and coefficient of variation for each batch of the AuNPs was provided by the manufacturer. In this paper, the average size of the AuNPs was 19.7 nm with 8% of coefficient of variation. Bovin serum albumin (BSA) were supplied by Sigma-Aldrich (Bornem, Belgium). Infliximab (Remicade®) was obtained from Janssen Biologics B.V. (Leiden, the Netherlands) and adalimumab (Humira®) was purchased from Abbvie (North Chicago, IL, USA). Anti-IFX monoclonal antibodies MA-IFX20G2, MA-IFX3D5 and MA-IFX6B7 were generated in the Laboratory of Therapeutic and Diagnostic Antibodies (KU Leuven, Belgium), as previously described (Van Stappen et al. 2015a, 2015b). Pooled serum, obtained from 30 healthy volunteers and de-identified serum samples from five IFX treated IBD patients, were taken in the framework of the VLECC study (B322201213950/S53684) after obtaining informed consent. In this paper, serum refers to pooled serum from healthy volunteers and serum sample refers to IFX treated patient serum. High binding 96-well plates were provided by Costar (Corning Inc., Corning, NY, USA). Phosphate buffer saline (PBS), 2-(N-morpholino)ethanesulfonic acid (MES) and sodium acetate buffers were 10 mM at pH 7.4, 50 mM at pH 6.0 and 10 mM at pH 4.5, pH 5.0 and pH 5.5, respectively unless otherwise specified.

2.2. Methods

2.2.1. FO-SPR platform and preparation of fiber probes

A detailed description of the FO-SPR biosensor platform can be found in [Supplementary information](#) and our previous publications (Arghir et al., 2015; Pollet et al., 2009).

2.2.2. Surface functionalization of the FO probes with antibodies

Gold coated optical fibers (FO probes) were functionalized with a carboxyl self-assembling monolayer (SAM) via overnight treatment at 4 °C in a 1 mM mixed solution of ethanol/carboxylic acid-SAM formation reagent (volume ratio of 9:1). FO probes were washed with ethanol after the overnight treatment and stored in MES buffer at 4 °C until use. SAM functionalized FO probes were immersed in 0.4 M EDC/0.1 M NHS mixture dissolved in MES buffer for 15 min to activate carboxylic groups of the SAM. Afterwards, the IFX specific monoclonal antibodies were immobilized on the FO surface for 30 min by covalent binding to the activated carboxylic groups. The monoclonal antibody, MA-IFX20G2, was immobilized on the FO probes as capture antibody. The FO probes were immersed twice in regeneration buffer (50 mM NaOH/1 M NaCl) for 30 s in order to remove antibodies that were not covalently bound to the surface. This was followed by immersion in blocking buffer (50 mM ethanolamine in PBS) for 8 min to deactivate unreacted carboxylic groups. An example of the typical FO-SPR sensorgram representing all the steps from this surface chemistry protocol for covalent immobilization of antibodies is illustrated in [Fig. 1](#). FO probes prepared as described were further used for detecting IFX spiked in buffer and serum (see [Section 2.2.4](#)) as well as for detecting IFX in patient serum samples (see [Section 2.2.5](#)).

2.2.3. Surface functionalization of AuNPs with antibodies

The detection antibodies (MA-IFX3D5 or MA-IFX6B7) were conjugated to AuNPs via physical adsorption. The protocol was

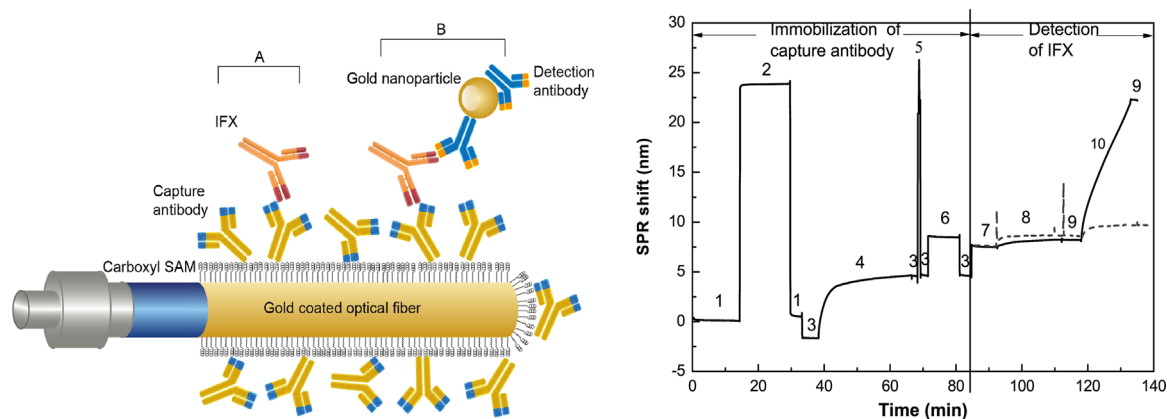


Fig. 1. Schematic overview of the sandwich bioassay. Left: a schematic representation of the EDC/NHS chemistry-based sandwich bioassay on FO probe surface. Carboxyl SAM was first formed on the surface of a gold coated FO. Capture antibodies were covalently immobilized on the surface through EDC/NHS activated carboxyl groups. IFX molecules were bound to the capture antibodies and the signal was enhanced by AuNPs functionalized with detection antibodies. (A) Label-free; (B) sandwich configuration. Right: typical FO-SPR sensorgram representing all the steps of EDC/NHS protocol for covalent immobilization of antibodies and IFX detection using sandwich bioassay approach. The dashed line on the detection part represents the detection of 10 $\mu\text{g/ml}$ of IFX using signal amplification with 20 $\mu\text{g/ml}$ of MA-IFX6B7 without AuNPs. (1) MES buffer; (2) EDC/NHS; (3) Sodium acetate buffer pH 5.5; (4) Capture antibody; (5) Regeneration buffer: 50 mM NaOH/1 M NaCl; (6) Blocking buffer: 50 mM ethanolamine in PBS; (7) PBS with 0.01% of Tween 20; (8) IFX; (9) PBS with 0.5% of BSA; (10) AuNPs.

based on a previously published paper (Jans et al., 2009) with several modifications as described here. The pH of the AuNPs was pre-adjusted at pH 9.2 using 0.2 mM of sodium carbonate. In a protein low-bind tube, antibodies were added to 800 μL of AuNPs (7×10^{11} particles/ml) to reach a final concentration of 5 $\mu\text{g/ml}$, and incubated on a rotator at room temperature for 20 min. Then, 560 μL of BSA (0.5% w/v) was added to stabilize the NPs. After an hour of rotation at room temperature, the NP solution was centrifuged at 7000 rpm and 20 $^{\circ}\text{C}$ for 30 min. After discarding the supernatant, containing unbound antibodies, NPs were finally re-suspended in PBS with 0.5% of BSA. In order to ensure reproducibility, the concentration of NPs was controlled to be the same as pre-functionalized NPs, by measuring the optical density (OD) of the NPs using a spectrometer (SpectraMax M2e, Molecular Devices, CA, USA). The OD of purchased AuNPs was 1, corresponding to the concentration of 7×10^{11} particles/ml. After functionalization and re-suspension in the buffer, AuNPs were adjusted to remain the same OD. In each assay, 150 μL of functionalized AuNPs were used. The prepared AuNPs were stored at 4 $^{\circ}\text{C}$ until use.

2.2.4. Establishing FO-SPR assay for IFX detection in buffer and serum

FO probes, functionalized with the IFX-specific capture antibody, were used for detecting IFX either directly or through a sandwich bioassay (Fig. 1). Direct detection of IFX was performed with 10 $\mu\text{g/ml}$ and 1 $\mu\text{g/ml}$ of IFX diluted in PBS with 0.01% Tween 20 for 20 min. The sandwich bioassay was established by using AuNPs pre-functionalized with MA-IFX3D5 or MA-IFX6B7 (here referred to as detection antibodies) in order to achieve signal amplification. The sandwich bioassay was performed in two different sample matrices: (i) IFX detection in buffer (PBS with 0.01% of Tween 20 spiked with IFX concentration series from 0 to 100 ng/ml: 0, 2.5, 5, 10, 25, 50, 75 and 100 ng/ml) and (ii) IFX detection in serum (100-fold and 200-fold diluted serum spiked with IFX concentration series from 0 to 75 ng/ml: 0, 2.5, 5, 10, 20, 25, 40, 50, 75 ng/ml). FO probes were functionalized with the capture antibodies as described in Section 2.2.2 and were immersed in IFX dilution for 15 min, followed by 20 min of signal amplification using functionalized AuNPs. The surface of FO probes was treated with regeneration buffer (two times 30 s) in between each IFX concentration in order to wash off the bound IFX and AuNPs. The serum was diluted with PBS containing 0.01% of Tween 20. An example of the typical FO-SPR sensorgram representing all

the steps of IFX detection through the sandwich bioassay is illustrated in Fig. 1, where significant increase in signal is shown with signal amplification by AuNPs. All SPR measurements were conducted at room temperature.

2.2.5. IFX quantification in IFX treated patients' sera using FO-SPR

For detecting IFX in infliximab treated patients' sera using FO-SPR, FO probes were functionalized with capture antibodies as described in Section 2.2.2 and the sandwich bioassay was similar to Section 2.2.4. Here, two concentrations of IFX spiked in 200-fold diluted serum (0 and 5 ng/ml) were first measured as a reference. Using the same FO probe, after removing bound IFX and AuNPs with the regeneration buffer, the IFX concentration was measured in patient serum (diluted 200- or 500-fold) followed by a signal amplification step with AuNPs. Five IFX treated patients' sera were tested and four repetitions were performed for each sample. For these experiments, MA-IFX3D5 was used as the detection antibody conjugated to AuNPs. IFX treated patients' sera were diluted in PBS with 0.01% of Tween 20.

2.2.6. IFX quantification in IFX treated patients' sera using ELISA

IFX in serum samples from IFX treated IBD patients was quantified using ELISA as previously described (Van Stappen et al., 2015a). Taking 150-fold diluted serum into account, the assay cut-off and lower limit of quantification are 0.2 $\mu\text{g/mL}$ and 0.5 $\mu\text{g/mL}$ of IFX, respectively. Each sample was measured three times at different days.

3. Results and discussion

3.1. Immobilization of IFX-specific antibody on the surface of FO probes

FO probes, functionalized with the IFX-specific capture antibody (MA-IFX20G2), were used in this paper for detecting IFX either directly at higher concentrations (10 and 1 $\mu\text{g/ml}$) or through a sandwich bioassay for detecting IFX at lower concentrations (between 2.5 ng/ml and 1 $\mu\text{g/ml}$). The sandwich bioassay was established by using AuNPs pre-functionalized with detection antibodies (MA-IFX3D5 or MA-IFX6B7) in order to achieve signal amplification (Fig. 1). These antibodies have been selected based on previously obtained results (Van Stappen et al. 2015a, 2015b, 2015c).

In order to develop a highly specific and sensitive FO-SPR based assay for IFX detection, experiments were first carried out to establish protocols for immobilization of IFX-specific capture antibody (MA-IFX20G2) on the sensor surface. As shown previously, the efficiency of antibody immobilization depends on several parameters, such as buffer pH, antibody concentration, ionic strength and reaction time. Thus, increased immobilization efficiency can be achieved in a buffer with low ionic strength and pH close to but smaller than the isoelectric point (pI) of the antibody (Pei et al., 2010; Pillet et al., 1994). Because the pI of the capture antibody, MA-IFX20G2, used in this study was unknown, buffers with low ionic strength (10 mM sodium acetate buffer) and with pH ranging from pH 4.5 to pH 5.5 were tested for covalent immobilization of this antibody at three different concentrations (10, 20 and 40 $\mu\text{g/ml}$).

The obtained results were analyzed with two-way Anova statistical test and subsequently Bonferroni correction to determine statistically significant difference between immobilization conditions. The maximum immobilization was achieved when using sodium acetate pH 5.5 for all three tested antibody concentrations (Fig. 2A). This can be due to the pI of the capture antibody being slightly higher than pH 5.5, resulting in positively charged antibodies and negatively charged carboxyl groups of SAM at pH 5.5. Moreover, under this condition, the detection of 10 $\mu\text{g/ml}$ IFX also resulted in the highest SPR shift (Fig. 2B). Based on the experimentally obtained SPR shifts and the statistical analysis, 20 $\mu\text{g/ml}$ of the capture antibody was immobilized on the FO surface in sodium acetate buffer pH 5.5 in all further experiments.

3.2. Controlling the non-specific binding on the surface of FO probes

Controlling the non-specific binding of target molecules and other sample components to the sensor surface is critical for the specificity of the assay. Therefore, series of experiments were carried out to test (i) non-specific binding of target molecules to the sensor surface, (ii) non-specific interaction of sample matrix components, such as serum, with the capture antibody on the sensor surface and (iii) non-specific interaction between detection antibodies, immobilized on AuNPs, and capture receptors on the sensor surface. Moreover, the specificity of monoclonal antibodies, employed in this study, has been tested by using another anti-TNF monoclonal antibody, namely adalimumab (ADM).

In order to demonstrate that there is no interaction between IFX and the sensor surface itself, FO probes were immersed in IFX

solution in the absence of capture antibodies. Thus, the sensor surface was prepared by activating carboxyl groups of the SAM layer and their subsequent deactivation using blocking buffer. Here, three different blocking buffers were tested, all capable of reacting with unoccupied carboxyl groups on the surface: 50 mM Tris in PBS, 50 mM ethanolamine in PBS and the commercial blocking solution supplied by Dojindo Laboratories (Kumamoto, Japan). These experiments resulted in no detectable SPR shift (as shown in Supplementary Fig. S2), irrespective of the type of blocking buffer used, which indicated that all three blocking buffers efficiently prevented undesired interaction between IFX and the sensor surface in the absence of IFX specific receptors.

Next, three blocking buffers were tested for their capacity to prevent the nonspecific binding of the components from the serum matrix. After immobilizing the capture antibody, FO probes were immersed in the blocking buffer, followed by immersion in 50-fold diluted serum or 50-fold diluted serum spiked with 10 $\mu\text{g/ml}$ of IFX. The biggest signal-to-noise ratio was obtained when using 50 mM ethanolamine in PBS (Fig. 3). The observed differences between Tris and ethanolamine buffers might be due to smaller size of ethanolamine molecules, resulting in easier interaction with unoccupied carboxyl groups among immobilized capture antibodies without blocking the binding sites of the antibodies. However, it is difficult to speculate why the specific signal was lower when using commercial blocking solution since the composition of this commercial reagent is unknown. Finally, because ethanolamine buffer also resulted in the smallest variability over different experiments, this blocking buffer was selected for the further work described in this paper.

Furthermore, the non-specific binding between functionalized AuNPs and the capture antibodies on the sensor surface was tested in a sandwich assay in the absence of IFX (0 $\mu\text{g/ml}$). The experiment was performed using AuNPs functionalized with two different detection antibodies, MA-IFX3D5 and MA-IFX6B7. The non-specific binding was negligible in the buffer (as seen in Fig. S3), irrespective of the detection antibody used (0.05 ± 0.02 nm and 0.1 ± 0.04 nm for MA-IFX3D5-AuNPs and MA-IFX6B7-AuNPs, respectively) and was very limited in 100-fold diluted serum (0.8 ± 0.04 nm and 0.3 ± 0.2 nm from MA-IFX3D5-AuNPs, respectively (data not shown)), thereby not interfering with detection of the lowest IFX concentration included in the assay (2.5 ng/ml). Therefore, both detection antibodies were utilized further in experiments.

As a final step in testing the specificity of the implemented bioassays, monoclonal antibodies were challenged against ADM,

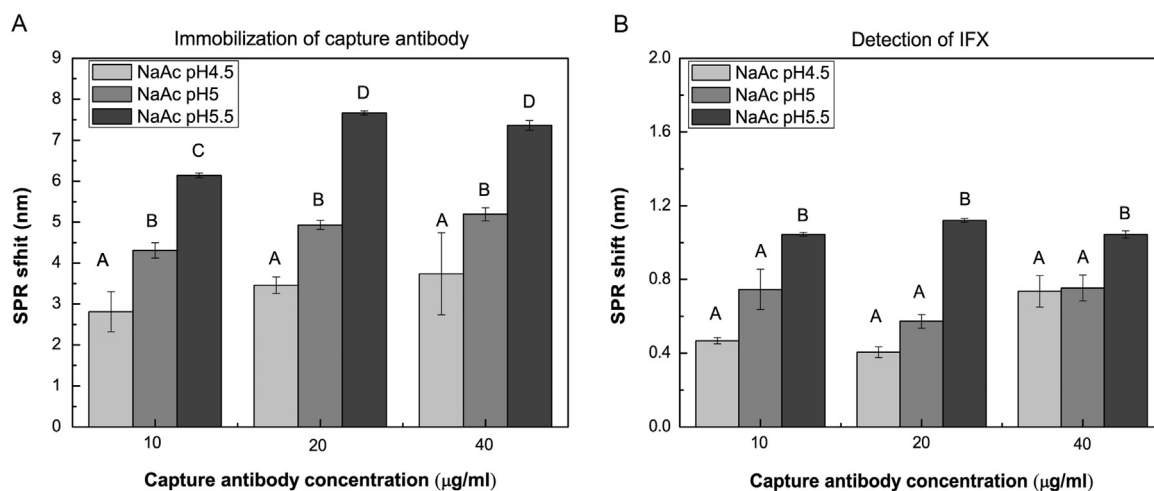


Fig. 2. Results of immobilization. Summary of immobilization shifts for 10, 20 and 40 $\mu\text{g/ml}$ of the capture antibody diluted in buffer with three different pH values (A), and corresponding SPR shifts for detection of 10 $\mu\text{g/ml}$ of IFX (B). Error bars represent standard deviation ($n=3$). Threshold letters (A, B, C and D) indicate statistically significant differences from Bonferroni correction.

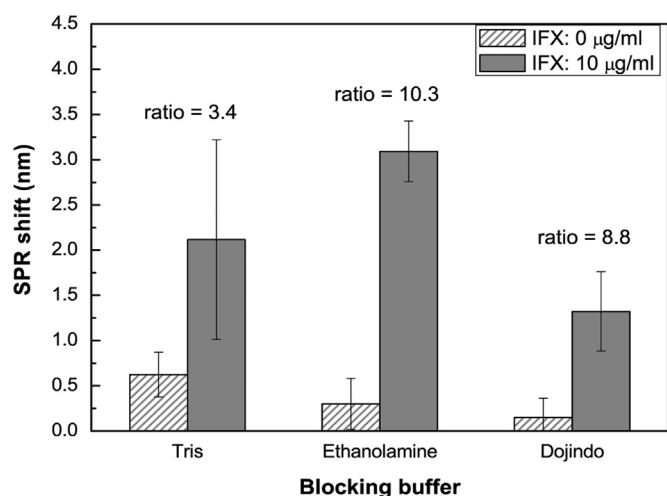


Fig. 3. Summary of SPR shifts obtained from testing blocking buffers. Non-specific binding test was performed in 50-fold diluted serum with functionalized FO probes in the absence of IFX. Specific signal was measured with 10 µg/ml of IFX spiked in the same serum dilution. Error bars represent standard deviations ($n=2$).

another anti-TNF biological (Billioud et al., 2011; Malottki et al., 2011). Although all three monoclonal antibodies employed here have been already described as IFX specific in other assays, experiments were performed on the FO-SPR platform as well to test whether the developed bioassay detects other anti-TNF biologicals. The non-specific signal was first measured in the absence of a target, followed by FO probe surface regeneration and measurement of 75 ng/ml of ADM diluted in buffer or 100-fold diluted serum. In this experiment, MA-IFX3D5 was used as detection antibody conjugated to the AuNPs. The obtained results revealed no significant difference between the blank serum and the one spiked with 75 ng/ml of ADM (Supplementary Fig. S4), demonstrating the specificity of selected monoclonal antibodies, as also previously shown in other assays (Van Stappen et al., 2015a, 2015b).

3.3. Detecting IFX spiked in buffer and serum using FO-SPR platform

Trough levels of IFX in patients' serum are commonly in the range of 0.5 to 10 µg/ml (Steenholdt et al., 2011). In order to avoid matrix effect, patients' samples are usually diluted at least 100-fold before testing, resulting in an IFX range of 5 to 100 ng/ml in the diluted serum samples. Because an SPR shift of 1 nm was

obtained when measuring 1 µg/ml of IFX diluted in buffer, the sensitivity of the direct immunoassay was considered as insufficient for detecting clinically relevant IFX concentrations. Moreover, building a sandwich assay with the addition of IFX-specific detection antibody only slightly improved SPR shift for additional 1 nm when using 10 µg/ml of IFX and 20 µg/ml of detection antibody (MA-IFX6B7) (Fig. 1 dashed line). Therefore, AuNPs were utilized for SPR signal enhancement, as similar successful applications have been previously shown by us and others (Law et al., 2011; Lyon et al., 1998; Pollet et al., 2011; Tran et al., 2013; Wang et al., 2010).

As expected, SPR signal enhancement was achieved by using AuNPs functionalized with either MA-IFX3D5 or MA-IFX6B7 (Supplementary Fig. S3). Using such established sandwich bioassay, a series of IFX concentrations, ranging from 0 to 100 ng/ml, were measured in PBS buffer with 0.01% of Tween 20. Measurements were conducted using the same FO probe for the entire range of IFX concentrations, which was attainable due to the removal of AuNPs and IFX in between subsequent steps using 50 mM NaOH/1 M NaCl as the regeneration buffer (Supplementary Fig. S3). The obtained average SPR shifts ($n=2$) were plotted as a function of IFX concentrations to generate two calibration curves, one for each of the detection antibodies (Fig. 4A). Calibration curves were fitted throughout the entire measured concentration range of IFX with nonlinear regression, one-site binding fitting, using Origin 8 (OriginLab, Northampton, US). Eq. (1), from the one-site binding fitting, was used to calculate the LOD of the established assays:

$$y = x \cdot A / (x + B) \quad (1)$$

where x represents the IFX concentration, y represents SPR shift, and A and B represent fitting parameters. To calculate LOD, the following equation can be used

$$\text{LOD} = \frac{(y(0) + 3\sigma)B}{A - (y(0) + 3\sigma)} \quad (2)$$

where $y(0)$ is the average SPR shift of non-specific binding, σ standard deviation of non-specific binding ($n=2$ for buffer and 6 for 100-fold diluted serum). Based on Eq. (2), the calculated LODs were 0.25 ng/ml (1.7 pM) and 0.51 ng/ml (3.5 pM) in buffer for MA-IFX3D5 and MA-IFX6B7, respectively.

The final assays were performed in 100-fold diluted serum spiked with IFX ranging from 0 to 75 ng/ml. The measurements were done similarly to the experiments performed in buffer with

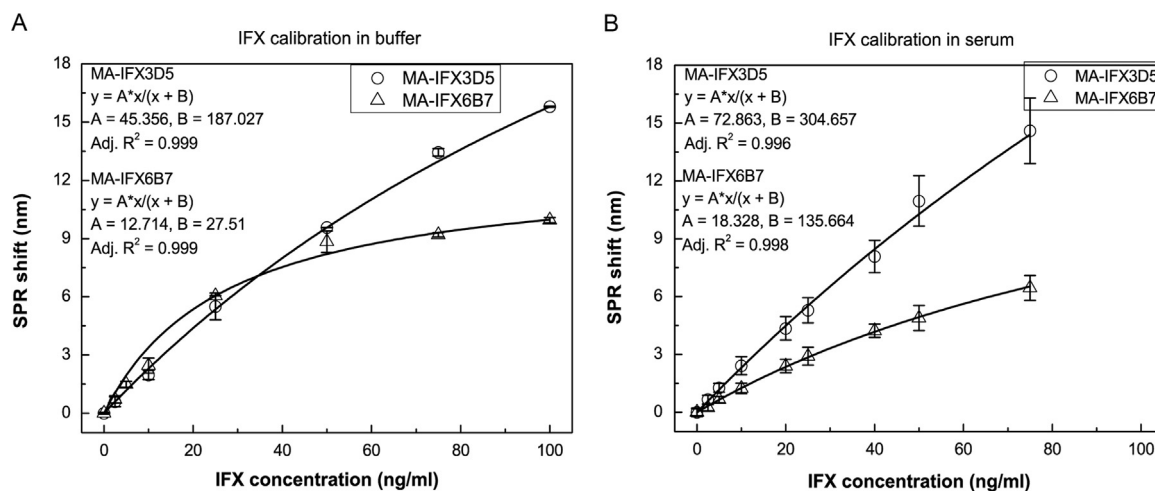


Fig. 4. Summary of calibration curves. Calibration curves obtained from series of IFX concentrations in buffer (A) and 100-fold diluted serum (B) when using AuNPs conjugated to MA-IFX3D5 and MA-IFX6B7 as labels for signal enhancement. A nonlinear regression (one-site binding) was fitted for both calibration curves. Fitting parameters are indicated on the left of the graph. Error bars represent standard deviations ($n=2$ for buffer and 6 for serum).

one FO probe used for the entire range of IFX concentrations and implemented regeneration steps in between each concentration. The obtained average SPR shifts ($n=6$) were plotted as a function of the IFX concentrations spiked in serum (Fig. 4B). The calculated LODs were 2.2 ng/ml (15 pM) and 5.3 ng/ml (37 pM) in 100-fold diluted serum, which correspond to 0.22 $\mu\text{g/ml}$ and 0.53 $\mu\text{g/ml}$ in whole serum for MA-IFX3D5 and MA-IFX6B7, respectively. To evaluate if the concentration of an unknown sample can be accurately determined by interpolating SPR signal into a calibration curve, analysis was carried out with the calibration curve obtained with MA-IFX3D5-AuNPs, as explained in [Supplementary information](#) (Supplementary Fig. S5). The difference between the actual and determined IFX concentrations from interpolation in calibration curve was less than 5%.

3.4. Validation of established FO-SPR bioassay with sera from IFX treated patients

The developed FO-SPR bioassay for IFX detection was finally evaluated by measuring IFX concentrations in sera of five IFX treated patients. All five serum samples (referred to as S1, S2, S3, S4 and S5) were first diluted 200-fold and then measured with the FO-SPR platform. AuNPs conjugated with MA-IFX3D5 were used for signal amplification in this assay, as this antibody resulted in better LOD compared to MA-IFX6B7 (see Fig. 4). Four measurements were carried out for each serum sample with two independently prepared batches of fibers and AuNPs, for estimating the inter assay variation. Subsequently, IFX concentrations were calculated from the calibration curve made in 200-fold diluted serum (Supplementary Fig. S6). Independently, the same serum samples were also tested with the in-house developed IFX ELISA (Van Stappen et al., 2015a). The obtained IFX concentrations from both assays are summarized in Table 1.

From all five tested serum samples from IBD patients, it appeared that the obtained SPR shifts for S2 were indistinguishable from those obtained from 0 ng/ml of IFX. A similar result was confirmed using ELISA. After de-blinding the samples, sample S2 appeared to be obtained from a patient naïve to IFX therapy, corroborating our findings. Because the concentration of IFX in S3 appeared to be out of the dynamic range of the used FO-SPR calibration curve when diluting samples 200-fold, S3 was further diluted to 500-fold. Nevertheless, the interpolation for this sample was obtained from the same calibration curve made in the 200-fold diluted serum, as this had no influence on the final concentration determination (Supplementary Fig. S7). The IFX concentrations from five serum samples were determined based on 200-fold diluted serum calibration curve as shown in Table 1. The coefficient of variation of the SPR shifts from four measurements of individual samples was less than 10%. Finally, the IFX concentrations measured with the newly developed FO-SPR assay and validated ELISA test had an excellent correlation (Fig. 5), as demonstrated with a Pearson correlation of 0.998 and an intraclass correlation coefficient (ICC) of 0.983.

Table 1
Determined concentrations of 5 serum samples from IBD patients using both ELISA and FO-SPR platform. Errors represent standard error of the mean.

Clinical sample	S1 ($\mu\text{g/ml}$)	S2 ($\mu\text{g/ml}$)	S3 ($\mu\text{g/ml}$)	S4 ($\mu\text{g/ml}$)	S5 ($\mu\text{g/ml}$)
ELISA ($n=3$)	3.4 ± 0.1	<0.2	19.2 ± 1.5	6.4 ± 0.6	4.0 ± 0.5
FO-SPR ($n=4$)	3.8 ± 0.1	<0.2	23.1 ± 1.7	6.2 ± 0.3	4.1 ± 0.2

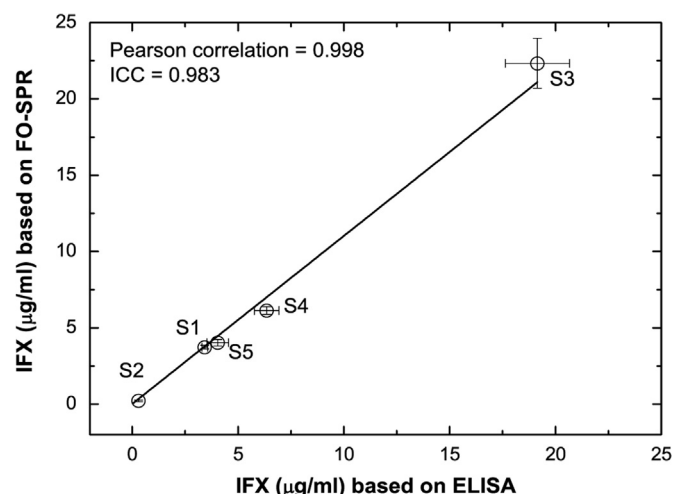


Fig. 5. Evaluation of the FO-SPR assay with serum samples from IBD patients. The determined IFX concentrations from FO-SPR platform were compared to IFX concentrations measured with the in-house developed ELISA. The Pearson correlation and ICC were determined to be 0.998 and 0.983, respectively. Error bars represent standard errors ($n=4$ for FO-SPR and 3 for ELISA).

4. Conclusions

In this paper we described the development of a fast and accurate bioassay for measuring IFX concentration in serum using the FO-SPR biosensor. To achieve this, selection of buffers and range of antibody concentrations was first studied for immobilizing in-house developed IFX specific antibodies on the sensor surface. Among tested conditions, 10 mM sodium acetate buffer with the pH value of 5.5 resulted in the best immobilization of capture antibody, particularly for 20 $\mu\text{g/ml}$ of antibody concentration. Next, a series of experiments were performed for controlling the non-specific binding of the target molecules, bioassay components and sample matrix to the sensor surface. These experiments demonstrated that IFX binds specifically to the sensor only in the presence of capture antibodies (MA-IFX20G2) as well as that all three antibodies used in this research have high specificity towards IFX and not towards other anti-TNF biologics. Moreover, among three tested blocking solutions, ethanolamine buffer appeared as the most suitable for detecting IFX in serum due to the maximal obtained signal-to-noise ratio.

Because a direct assay was insufficient for detecting low concentrations of IFX (below 1 $\mu\text{g/ml}$), the SPR signal was amplified using AuNPs conjugated to two different detection antibodies (MA-IFX3D5 and MA-IFX6B7). Using the established sandwich immunoassay, calibration curves were made in buffer and 100-fold diluted serum, with the best obtained LOD of 0.3 ng/ml (1.7 pM) and 2.2 ng/ml (15 pM) respectively, when using MA-IFX3D5 as detection antibody. Final evaluation of the developed FO-SPR bioassay was performed using five serum samples from IFX treated IBD patients, analyzed using ELISA and FO-SPR. These results revealed excellent agreement between the FO bioassay and the clinically validated ELISA, showing excellent correlation (Pearson correlation of 0.998 and ICC of 0.983) and inter-coefficient of variation being less than 10%.

Although the total time of the presented assay is 2.5 h (including immobilization of the capture antibody), it is envisioned that fiber probes will be pre-functionalized for future use in a hospital together with kinetic analysis, which will reduce the assay time to less than 20 min. Therefore, individual tests on patient samples at point-of-care become feasible. In addition, the FO-SPR biosensor is fully automated and can be run as a single measurement or with 4 parallel FO sensors. Thereby, the developed

bioassay combined with characteristics of the FO-SPR platform demonstrates its potential as a POC diagnostic tool that can be used for determining biological drugs in serum samples from patients.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at <http://dx.doi.org/10.1016/j.bios.2015.11.087>.

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