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A rapid immunoassay for detection of infliximab in whole blood using a fiber-optic SPR biosensor

Jiadi Lu[1], Dragana Spasic[1], Filip Delport[1,4], Thomas Van Stappen[2], Iris Detrez[2], Devin Daems[1], Séverine Vermeire[3], Ann Gils[2] and Jeroen Lammertyn[1,4,*]

[1]BIOSYST-MeBioS, KU Leuven, Leuven, Belgium

[2]Laboratory for Therapeutic and Diagnostic Antibodies, KU Leuven, Leuven, Belgium

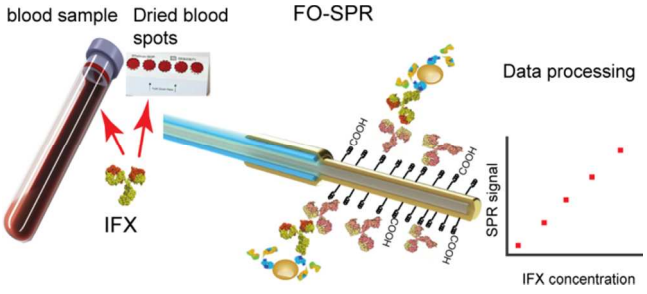
[3]Division of Gastroenterology, UZ Leuven, Leuven, Belgium

[4]Fox Diagnostics, Temse, Belgium

*jeroen.lammertyn@kuleuven.be

KEYWORDS point-of-care, surface plasma resonance, fiber-optic, infliximab, whole blood, dried blood spots

ABSTRACT: Monitoring the concentration of a therapeutic drug antibody, infliximab (IFX), is recommended for enhancing its efficacy in patients with inflammatory bowel disease (IBD). However, IFX concentrations are currently determined in patients' serum/plasma, which requires sample preparation from blood, hence hampering the turnaround time. In this paper, we present a short immunoassay (10 minutes) using fiber-optic SPR (FO-SPR) biosensor for detection of IFX spiked in 100-fold diluted serum, plasma and whole blood. The calculated limits of detection (LOD) based on calibration curves were 1.42, 1.00 and 1.34 ng/mL, respectively, which coincides with expected IFX concentrations in diluted samples from IBD patients. A linear correlation was established among different matrices, indicating that the matrix effect was insignificant. The established point-of-care (POC) FO-SPR bioassay was also used to measure IFX in 100-fold diluted extracts of dried blood spots (DBS) and LOD achieved was below 2 ng/mL. Although DBS might be ideal for POC, this is the first report of using an SPR biosensor for measuring DBS samples. Finally, the POC FO-SPR immunoassay was validated by using matching serum and plasma samples from five IBD patients. A Pearson correlation of 0.968 was obtained between serum and plasma samples. IFX concentrations determined with FO-SPR were compared to a clinically validated ELISA, resulting in excellent Pearson correlation and intraclass correlation coefficient, both being 0.99 for serum and plasma samples. In conclusion, this paper demonstrates that our FO-SPR biosensor can be used as a true POC diagnostic tool for determining IFX concentrations in variety of matrices.



Infliximab (IFX) is a chimeric monoclonal antibody used in treatment of patients with moderate-to-severe inflammatory bowel diseases (IBD). Monitoring the IFX trough concentration (defined as the lowest IFX concentration in patient's blood immediately prior to the next infusion) is recommended for enhancing the therapeutic outcome of IBD patients, which is mostly done using an enzyme linked immunosorbent assay (ELISA). However, several significant disadvantages make ELISA unsuitable as a point-of-care (POC) technique: (i) requirement for well-trained staff to run the tests, (ii) relatively long time-to-result (approximately 2 hours) and (iii) the need for multiple patient samples to be collected in a central laboratory before the test can be executed. Thus, immediate dose adaption with ELISA tests is impossible. A recently reported lateral flow-based immunoassay has important advantages such as fast detection time (less than 20 minutes) and single sample testing [1,2]. However, either serum or plasma samples are still required for applying this technique. Therefore, an inexpensive platform that allows for single sample testing with a short detection time, good accuracy and minimum sample preparation is desirable to facilitate POC testing.

A sandwich immunoassay has recently been developed for IFX detection in serum using an in-house developed fiber-optic surface plasmon resonance (FO-SPR) biosensor, which is a fully automated multi-channel platform with inexpensive and disposable FO-SPR sensors [3]. However, despite all these advantages, the described method still required preparation of serum samples by centrifugation. Establishing direct IFX measurements in whole blood, such as blood from finger prick, while using the same FO-SPR platform, would eliminate the need for sample preparation, thereby making this a true simple-to-use POC with reduced turnaround time. Fur-

thermore, direct measurements in blood could potentially allow patients to collect blood samples at home through dried blood spot (DBS) sampling. In recent years, there has been growing interest in DBS sampling for therapeutic drug monitoring [4-6], as it is a simple, convenient and flexible sampling method for patients. Importantly, the DBS samples can be easily transported to a laboratory for further testing. In this way, regular monitoring of the drug level could be arranged without patients visiting the hospital.

In this paper, we present the development of an advanced FO-SPR immunoassay with 3 distinct features: (i) fast assay time, (ii) clinically relevant sensitivity and (iii) capacity for detecting IFX in serum, plasma, citrated whole blood (used in place of the finger prick blood) and DBS. Starting from the previously established bioassay [3], several aspects have been improved for maximizing signal-to-noise ratio in different matrices and establishing a true POC testing concept. The developed immunoassay was validated using clinical samples (both serum and plasma) from five IFX treated IBD patients. This paper demonstrates the versatility and robustness of the FO-SPR biosensor that can be used as a POC diagnostic tool in various types of patient samples.

EXPERIMENTAL SECTION

Buffers and reagents. All buffer reagents were obtained from Sigma-Aldrich (Diegem, Belgium), unless stated otherwise. All solutions were prepared with deionized water purified by a Milli-Q Plus system (Millipore, Marlborough, MA, USA). Acetone and acetic acid were purchased from Chemlab (Zedelgem, Belgium). Tween 20 and Tween 80 was provided by AppliChem GmbH (Darmstadt, Germany). Carboxylic acid-SAM formation reagent was produced by Dojindo Laboratories (Kumamoto, Japan). 1-ethyl-3-[3-dimethylaminopropyl] carbodiimide (EDC), N-hydroxysuccinimide (NHS) and Superblock™ (PBS) Blocking Buffer were obtained from Thermo Fisher Scientific (Erembodegem, Belgium). AuNPs EMGC20 with an average diameter of 20 nm were provided by BBI Solutions (Cardiff, UK). Bovin serum albumin (BSA) was supplied by Sigma-Aldrich (Diegem, Belgium). Infliximab (Remicade®) was obtained from Janssen Biologics B.V. (Leiden, the Netherlands). Anti-IFX monoclonal antibodies MA-IFX20G2 and MA-IFX3D5 [7] were generated in the Laboratory of Therapeutic and Diagnostic Antibodies (KU Leuven, Belgium). Human sodium citrate-treated whole blood (will be referred to as whole blood in the paper), plasma and pooled serum from healthy donors were purchased from Valley Biomedical (Winchester, USA). Human whole blood was collected in sodium citrate coated tubes and plasma was processed from this whole blood. Filter paper (903 Protein saver card) was purchased from Sigma-Aldrich (Diegem, Belgium). De-identified serum and plasma samples from five IFX-treated IBD patients were taken in the framework of the Vlaams Erfelijkheidsonderzoek crohn en colitis ulcerosa (VLECC) study (B322201213950/S53684) after obtaining informed consent from the patients. 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 5.5, respectively unless otherwise specified.

FO-SPR platform and preparation of FO-SPR sensors. A detailed description of the FO-SPR biosensor and preparation of FO-SPR sensors can be found in supporting information (SI) Figure S1 A and our previously reported publications [3,8]. Although all the results in this paper were obtained using the platform illustrated in Figure S1 A, a more compact and user-friendly FO-SPR platform, has been developed in the group for the POC use, as shown in Figure S1 B. When previously published IFX-specific bioassay[3] was implemented on this platform, excellent agreement was achieved with the published results (data not shown), revealing the great potential of this fully integrated FO-SPR device.

Detection of IFX spiked in 10-fold diluted whole blood. A calibration curve in 10-fold diluted whole blood was established using the previously developed FO-SPR sandwich bioassay for detection of IFX [3]. In brief, gold-coated FO-SPR sensors were functionalized overnight with a carboxyl self-assembling monolayer (SAM). Carboxyl groups were activated by EDC/NHS (0.4 M/0.1 M) chemistry for covalently binding the MA-IFX20G2 capture antibodies, supplied in a final concentration of 20 μg/mL. After removing non-covalently bound capture antibodies with regeneration buffer (10 mM glycine/HCl pH 2), the unoccupied carboxyl groups were deactivated using a blocking buffer (Superblock PBS). To obtain a calibration curve, the FO-SPR sensor was immersed for 15 minutes in the range of IFX concentrations (0, 2.5, 5, 10, 20, 40, 80 and 100 ng/mL) prepared in 10-fold diluted whole blood, followed by 20 minutes of signal amplification using AuNPs conjugated with detection antibody. In this paper, IFX and blood were diluted using PBS with 0.01% of Tween 20. Storage buffer of AuNPs was PBS with 0.5% of BSA, unless otherwise stated. An overview of the bioassay is indicated in SI Table S1A. The process of functionalization of the AuNPs with the detection antibody, MA-IFX3D5, was the same as described in our previous publication [3]. In contrast to the previous publication, in this paper each IFX concentration was measured with an individual FO-SPR sensor.

Preparation and extraction of DBS samples. For preparation of 100-fold diluted DBS samples, IFX was first 10-fold diluted from the stock solution in whole blood (by adding 10 μL of IFX into 90 μL of whole blood), resulting in the range of IFX concentrations (0, 0.25, 0.5, 1, 2, 4, 8, 10 μg/mL). 45 μL of this dilution was applied onto a disc of a filter paper (903 Protein saver card, Whatman). The DBS samples were allowed to dry overnight at room temperature. Subsequently, diameter of 6 mm disks were punched out from the filter paper and placed into an Eppendorf tube containing 240 μL of extraction buffer (PBS with 0.1% of Tween 80). This was placed on a shaker (Thermomixer comfort, Eppendorf) for 1 hour set at 300 rpm and 21 °C. The samples were centrifuged (Microstar 17R, VWR) for 5 minutes at 13000 rpm (16249 g) and used immediately after preparation. The blood volume from 6 mm disk was approximately 10 μL, which led to the first 25-fold dilution in the 240 μL of extraction buffer. To reach final concentrations of IFX in DBS, being 0, 2.5, 5, 10, 20, 40, 80 and 100 ng/mL, the DBS samples were additionally diluted 4-fold using the detection buffer (PBS with 0.01% of Tween 20). In this paper, we refer to this type of DBS sample as a spiked DBS sample.

To estimate the extraction efficiency, DBS samples without IFX were also prepared. For this, 10 μL of detection buffer was added into 90 μL of whole blood instead of IFX. The extraction procedure was the same as described above resulting in 25-fold diluted whole blood. The extracted blood was additionally diluted 4-fold with the detection buffer and spiked

with IFX in order to obtain the same series of final concentrations as above (0, 2.5, 5, 10, 20, 40, 80 and 100 ng/mL). In the context of this paper, we refer to this type of DBS sample as a reference DBS sample.

Establishing a POC FO-SPR bioassay for IFX detection. A POC FO-SPR bioassay was established following the protocol for functionalization with the capture antibodies as described in the previous section. Functionalized FO-SPR sensors were subsequently immersed in IFX solution (i.e. samples spiked with IFX) for 5 minutes, AuNP storage buffer (PBS with 0.5% of BSA) for 3 minutes and AuNP solution for 5 minutes, reducing the total detection time to 13 minutes (as seen in SI Table S1B). This shortened detection assay was used to obtain calibration curves in 10-fold diluted whole blood, 100-fold diluted whole blood, serum, plasma and DBS extracts, spiked with final IFX concentrations ranging from 0 to 100 ng/mL. For each calibration curve, four repetitions were included, which were generated with two batches of independently prepared FO-SPR sensors and AuNPs.

Validation of the POC FO-SPR bioassay using serum and plasma samples from IFX-treated patients. The POC FO-SPR bioassay was validated using five IFX-treated patient samples, with matching serum and plasma samples from each patient. Three samples were 100-fold diluted. Due to higher IFX concentrations in the other two samples, one of them was 150-fold diluted and the other one was 300-fold diluted. Three repetitions were measured for each sample.

IFX quantification in serum and plasma samples from IFX-treated patients using ELISA. IFX in serum samples from IFX-treated IBD patients was quantified using ELISA as previously described [9]. Each sample was measured three times on different days.

Data analysis. To assess the influence of FO-SPR sensors and AuNPs that were generated on different days, results were analyzed using a randomized block design in JMP Pro12 (SAS Institute Inc., Cary, USA). For investigation of the sample matrix effect, correlations were made between calibration curves prepared in different matrices. A 95% confidence interval (95% CI) for slope was calculated using linear fitting in Origin® 8 (OriginLab, Northampton, USA).

RESULTS AND DISCUSSTIONS

FO-SPR bioassay for detection of IFX in 10-fold diluted whole blood. Detection of IFX trough levels is usually conducted in serum or plasma, which requires blood sample processing, leading to a longer time to result. Therefore, performing bioassays directly in whole blood, such as from a finger prick, would create a significant improvement in the disease management of IBD patients. In order to establish such bioassay in this paper, venous citrated whole blood was used as a matrix as obtaining blood from a finger prick was not feasible. Whole blood was diluted 10-fold and spiked with a series of IFX concentrations, ranging from 0 to 100 ng/mL. Starting from the previously established FO-SPR bioassay for IFX detection in serum [3], two major modifications were introduced to minimize the matrix effect from whole blood: (i) calibration curves were generated by using an individual FO-SPR sensor for each concentration, in contrast to one FO-SPR sensor used previously for a series of IFX concentrations and (ii) Superblock PBS was introduced as a blocking buffer, resulting in less than 0.1 nm shift for the non-specific binding (n = 4, measured by immersing the FO-SPR sensor functionalized with capture antibody in 10-fold diluted whole blood in the absence of the target, followed by a AuNP amplification step). To generate a calibration curve, the obtained average SPR shifts (n = 2) were plotted as a function of the IFX concentrations (Figure 1) and fitted with nonlinear regression (one-site binding fitting) using Origin® 8 (OriginLab, Northampton, US). The limit of detection (LOD) was determined as explained in SI Section 1.3, and equaled 1.12 ng/mL with a coefficient of variation (CV) below 10%.

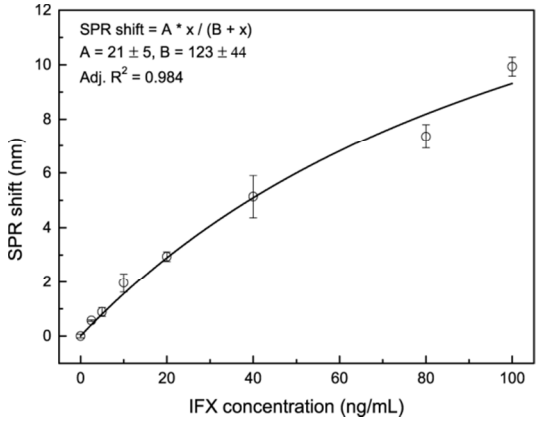


Figure 1. IFX calibration curve in 10-fold diluted whole blood measured with the FO-SPR bioassay. Unless otherwise stated, the equations displayed in all the graphs refer to the one-site binding fitting equation x stands for IFX concentration in ng/mL. The errors are standard deviations based on two repetitions.

Accelerated POC FO-SPR bioassay for detection of IFX in 10-fold diluted whole blood. Although the obtained results demonstrated for the first time measurements of IFX directly in whole blood, the total detection time was approximately 40 minutes (as seen in Table S1 A), which is not fully compatible with a POC testing concept. In order to further shorten the FO-SPR bioassay, the IFX incubation time and AuNP amplification step were reduced from 15 and 20 minutes, respectively to 5 minutes each, resulting in total detection time of 13 minutes (time of the assay corresponds to the time from the moment that the FO-SPR probe was immersed in IFX solution, as seen in Table S1 B). Using the established POC bioassay, a series of IFX concentrations (ranging from 0 to 100 ng/mL) was spiked in 10-fold diluted whole blood and measured. The SPR shifts obtained from AuNP binding were plotted as a function of IFX concentrations to generate a calibration curve (will be referred to as method 1 in this paper, Figure 2 A). Based on four repetitions, the calculated LOD was 0.75 ng/mL with an average CV value below 12%. All the measurements were performed in a random manner, meaning that IFX concentrations were not measured in ascending or descending order.

Since binding curves of the AuNPs showed a linear behavior within the first 2 minutes (SI Figure S2), this part of the curve was used to perform a slope analysis. The obtained values of the slope were further plotted as a function of IFX concentrations to generate a calibration curve (will be referred to as method 2 in this paper, Figure 2 B), which resulted in an LOD value of 0.9 ng/mL with an average CV below 18%. Although the CV value for method 2 was slightly higher compared to method 1, it is still acceptable as it lies below 20% [10]. Moreover, the obtained LODs from the two methods were

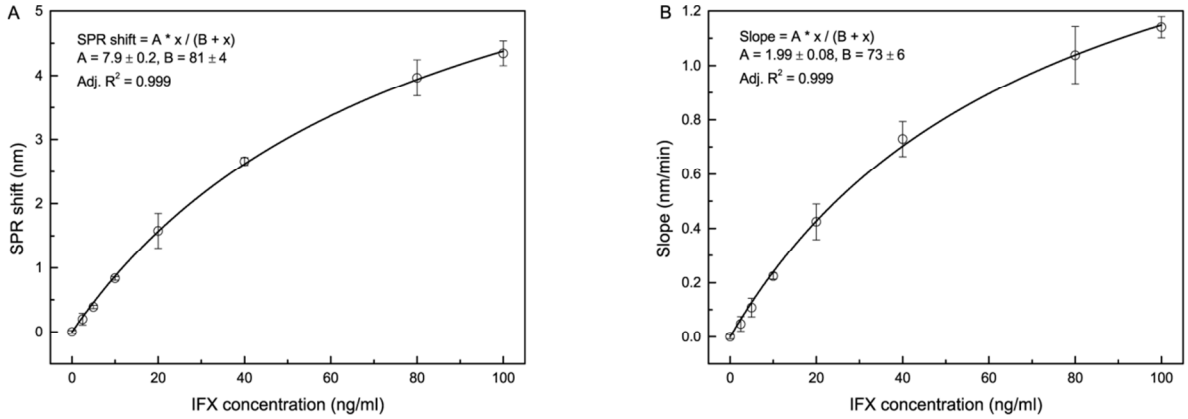


Figure 2. Calibration curves in 10-fold diluted whole blood with the POC FO-SPR immunoassay, using method 1 (A) and method 2 (B). The error bars are standard deviations based on four repetitions.

comparable, suggesting that the detection time of the bioassay can be further reduced by 3 minutes, towards a total detection time of 10 minutes.

Measurements of IFX concentrations in whole blood have never been previously reported in the literature, mainly for two reasons. First, red blood cells and platelets typically rupture when handling blood samples, causing clotting and an increase in non-specific absorption to the sensor surface, making it more challenging to detect target molecules. In this paper, the non-specific absorption from blood on the sensor surface was minimized by using Superblock PBS [11,12]. Second, IFX trough concentrations in whole blood are expected to be lower than in serum or plasma. In general, the serum/blood ratio is approximately 45%. Therefore, IFX trough concentrations in whole blood are approximately 45% of those in serum, which is in the range of 0.23 to 4.5 μg/mL (based on trough concentrations in serum samples, ranging from 0.5 to 10 μg/mL). This suggests that detection of IFX in whole blood requires a more sensitive assay than in serum. In this paper, the concentrations encompassed by the calibration curve were 2.5 to 100 ng/mL in 10-fold diluted blood, which corresponds to 0.025 to 1 μg/mL in whole undiluted blood. Therefore, in order to achieve detection of IFX in the clinically relevant range, the dilution factor was further adjusted as discussed in the next section of the paper.

POC FO-SPR bioassay for detection of IFX in 100-fold diluted serum, plasma and whole blood. To measure IFX concentrations in the clinically relevant range (i.e. 0.5 to 10 μg/mL in serum samples and 0.23 to 4.5 μg/mL in whole blood), samples need to be diluted 100-fold in order to fall in the dynamic range of the POC FO-SPR bioassay (0 and 100 ng/mL). Therefore, IFX concentrations, ranging from 0 to 100 ng/mL, were spiked in 100-fold diluted serum, plasma and whole blood and quantified with the POC FO-SPR bioassay. All the concentrations were measured in a random manner. By using method 1 and 2, calibration curves were generated for all three matrices (Figure 3 A and B). The data were fitted using a 'one-site binding' model [13] with the model parameters as indicated in Figure 3. The LOD values in 100-fold diluted serum, plasma and whole blood were respectively 1.05, 1.00 and 1.05 ng/mL obtained with method 1 and 1.42, 1.00 and1.34 ng/mL for method 2. Based on these results, it is evident that the obtained LODs were comparable for all three matrices irrespective of the used method. The CVs were below15% for both methods.

To investigate whether the FO-SPR sensors and AuNPs prepared on different days had an impact, all the results were analyzed with a randomized block design in JMPPro12 (SAS Institute Inc., Cary, USA). For all the calibration curves, the difference between the results measured on two independent days was statistically insignificant, implying that there was no statistical difference between two batches of FO-SPR sensors and AuNPs (data not shown). This further suggested that the FO-SPR biosensor does not require a new calibration curve to

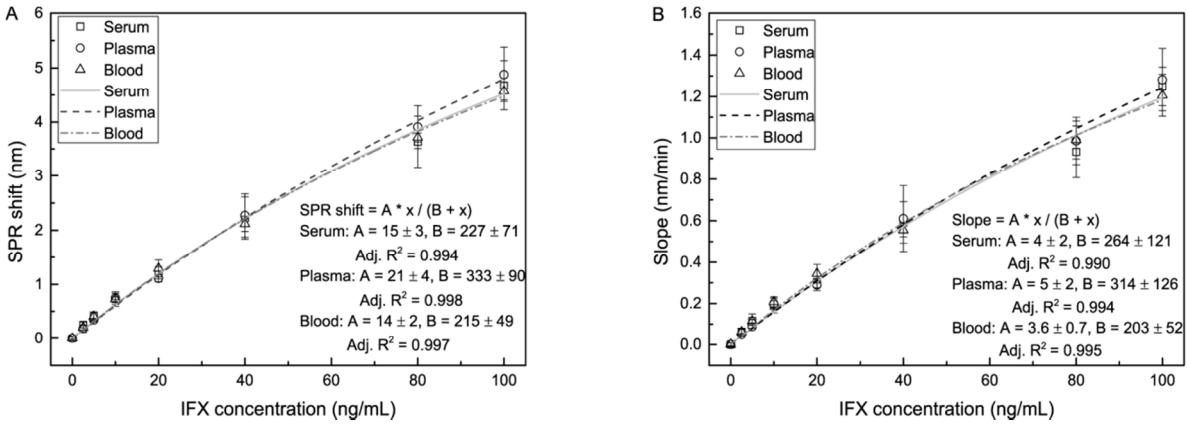


Figure 3. Calibration curves obtained for 100-folded diluted serum, plasma and whole blood based on method 1 (A) and method 2 (B) using POC FO-SPR bioassay. Four repetitions were included for each curve and the error bars are standard deviations.

be generated for each new batch of FO-SPR sensors or AuNPs that is being produced. Therefore, it is sufficient to perform only one control measurement (i.e. no target) and one concentration of IFX and compare the obtained values to the established calibration curve.

POC FO-SPR bioassay for detection of IFX in 100-fold diluted DBS. Capillary blood collected by finger-prick in the form of DBS might be ideal for POC as patients can perform sample collection by themselves at home and requirements for transportation of the DBS samples are minimal. However, mainly two concerns have made this practice difficult so far: (i) bursting of red blood cells after whole blood is dried, which can result in higher non-specific interaction between the ruptured cell fragments and the sensor surface and (ii) altered structure of analytes due to the drying process, which can influence the efficiency of the analytes entering the extraction buffer and induce increased variation of the extraction efficiency. The most frequently reported technologies for detection of DBS samples include ELISA [14], high-pressure liquid chromatography (HPLC) [15] and liquid chromatography-tandem mass spectrometry (LC-MS/MS) [16]. This is the first time that an SPR biosensor has been deployed for measuring DBS samples.

To develop a consistent and reproducible method for DBS sample preparation, a protocol was optimized as explained above. In order to determine the extraction efficiency of the developed protocol and assess the potential of using the FO-SPR platform in combination with DBS samples, IFX concentrations were measured in 100-fold diluted reference and spiked DBS samples. Here, a reference DBS sample refers to whole blood spiked with IFX *after* extraction through the filter paper, while a spiked DBS sample refers to whole blood spiked with IFX *prior* to extraction through the filter paper (see Experimental section for more details). Based on the obtained calibration curves when using the POC FO-SPR bioassay (Figure 4), the LOD values were 1.17 (spiked DBS) with a CV of 18% and 1.39 (reference DBS) ng/mL with a CV of 20% for method 1 and 1.83 (spiked DBS) with a CV of 17% and 1.59 (reference DBS) ng/mL with a CV of 20% for method 2 (n = 4).

The extraction efficiency is usually estimated by calculating the ratio of the analyte concentration from DBS samples and serum/plasma. However, in this paper, it was calculated as ratio of the analyte concentration (i.e. obtained SPR shifts for each IFX concentration) between spiked and reference DBS samples. The ratio was first individually calculated for each of the seven IFX concentrations (ranging from 2.5 to 100 ng/mL). The individual ratio values were subsequently used for calculating the average value, resulting in 0.98 when using method 1 and 0.97 for method 2. These results demonstrated that: (i) the drying process of blood did not affect the extraction of IFX or its interaction with the antibodies used in the FO-SPR bioassay and (ii) the distribution of IFX on the DBS paper was uniform since blood with and without IFX was spread out on the paper and only part of it was taken for extraction process. Similar values for extraction efficiency were also obtained when performing a correlation analysis between calibration curves from the reference and spiked DBS samples (SI Figure S3 A and B). This resulted in the slopes of 0.99 (95% CI slope: 0.99 ± 0.02) for for method 1 and 0.98 (95% CI slope: 0.99 ± 0.04) for method 2, with the Pearson correlation of 0.998 and 0.995, respectively.

Comparison of the POC FO-SPR bioassay performance in different matrices. Next, to investigate the impact that different matrices might have on measuring IFX concentrations with the POC FO-SPR bioassay, correlations were calculated between calibration curves obtained for 100-fold diluted whole blood, serum, plasma and DBS. The results are presented in Table 1 when using method 1 and in Support information Table S2 for method 2. For a give cell shown in the table, a correlation was obtained by using the matrix from the column as the x axis and the matrix from the row as the y axis. The linear equation of the fitted curve, 95% CI of the slopes and Pearson correlation are included in the tables.

When comparing all the results from 100-fold diluted serum, plasma and whole blood (indicated as bold in tables), the slope values were approximately 1 for both method 1 and 2. This suggested that for a given dilution factor, the SPR signal was almost identical regardless of the matrix, i.e. that the matrix effect from 100-fold diluted serum, plasma and whole blood was insignificant. A summary of linear regression analysis can be seen in SI Figure S4.

When the SPR signals from 100-fold diluted whole blood/serum/plasma were compared to those from spiked DBS samples, slope values of approximately 1.1 were obtained, suggesting a nearly 10% higher signal for 100-fold diluted blood/serum/plasma (Table 1 and SI Figure S5). This difference could be attributed to two different factors: (i) a matrix effect of DBS samples due to bursting of the blood cells during the drying process, which may interfere with the interactions between IFX and capture antibody/AuNP, thereby reducing the SPR signal and (ii) the calculation of dilution factor

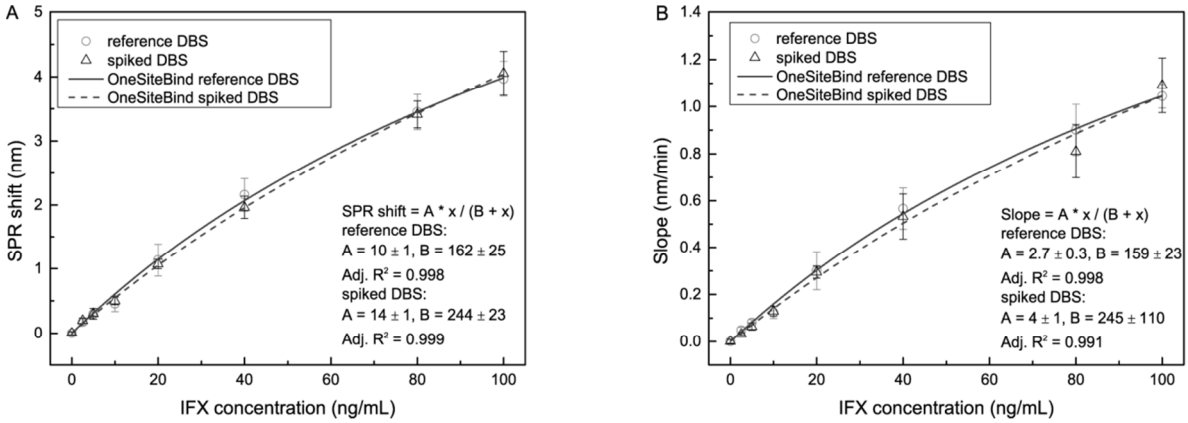


Figure 4. Calibration curves generated in 100-fold diluted reference DBS and spiked DBS samples based on method 1 (A) and method 2 (B). The error bars are standard deviations based on four repetitions.

Table 1. Correlations between calibration curves based on method 1 in various matrices. Linear regressions were fitted using calibration curves in the column as *x* axis and calibration curves in the row as *y* axis.

y-axis / x-axis	100-fold diluted whole blood	100-fold diluted serum	100-fold diluted plasma	100-fold diluted spiked DBS
100-fold diluted whole blood		**y = 1.01x – 0.02 95% CI: 0.04 Pearson: 0.999**	**y = 1.08x - 0.09 95% CI: 0.04 Pearson: 0.998**	y = 0.91x - 0.05 95% CI: 0.02 Pearson: 0.999
100-fold diluted serum	**y = 0.99x + 0.01 95% CI: 0.04 Pearson: 0.999**		**y = 1.07x – 0.07 95% CI: 0.04 Pearson: 0.999**	y = 0.93x - 0.07 95% CI: 0.02 Pearson: 0.999
100-fold diluted plasma	**y = 0.93x + 0.07 95% CI: 0.04 Pearson: 0.999**	**y = 1.00x + 0.01 95% CI: 0.1 Pearson: 0.996**		y = 0.88x - 0.01 95% CI: 0.02 Pearson: 0.999
100-fold diluted spiked DBS	y = 1.10x + 0.01 95% CI: 0.04 Pearson: 0.999	y = 1.10x + 0.06 95% CI: 0.06 Pearson: 0.998	y = 1.18x - 0.02 95% CI: 0.06 Pearson: 0.998	

during DBS sample preparation - although the volume of blood from the 6 mm DBS disk was assumed to be 10 µL, in practice, however, this could be less, suggesting that the final dilution factor in DBS sample preparation can be potentially greater than 100-fold.

Validation of the POC FO-SPR bioassay with plasma and serum samples from IFX treated patients. The developed POC FO-SPR bioassay was validated using matching serum and plasma samples from five IFX-treated IBD patients. The same serum and plasma samples were also tested using a clinically validated ELISA, based on the assay described in [9]. Taking 150-fold diluted serum/plasma into account, the ELISA cut-off and lower limit of quantification were 0.2 µg/mL and 0.5 µg/mL of IFX, respectively.

Measurements were first conducted with five serum samples (referred to as S1, S2, S3, S4 and S5) diluted 100-fold. Due to the high SPR shifts from S1 and S5, these two samples were further diluted 150- and 300-fold, respectively. IFX concentrations from serum samples were calculated by interpolating the obtained SPR signals into the calibration curve, which was made using the series of IFX concentrations spiked in 100-fold diluted serum. Although dilution factors of S1 and S5 were different from the calibration curve, it was demonstrated in our previous publication [3] that this has no influence on determining IFX concentrations. Next to this, IFX was quantified in the five plasma samples (referred to as P1, P2, P3, P4 and P5) from the same patients. Because the difference between serum and plasma is only in the presence of clotting factors in plasma but not in serum, similar IFX concentrations are expected in both matrices from the same patient. Therefore, the dilution factors of the plasma samples were kept the same as for the serum samples and the calibration curve was made using 100-fold diluted plasma spiked with series of IFX concentrations.

The measured IFX concentrations in serum and plasma samples were used for establishing the correlation between these two matrices (SI Figure S6). As seen from the figure, good Pearson correlations of 0.959 and 0.968 were obtained for method 1 and method 2, respectively. Slopes in the correlations were smaller than 1 for both methods, which indicated that IFX concentrations in the plasma samples were slightly lower than those in the corresponding serum samples. This can be due to the clotting factors in plasma that make these samples less stable compared to serum concerning handling and storage.

Finally, IFX concentrations measured with the POC FO-SPR bioassay were compared to those measured with ELISA, as seen in Figure 5. FO-SPR method 2 was selected for validating the POC FO-SPR bioassay with the ELISA since it gave very similar IFX concentrations for both serum and plasma patients' samples as method 1 (demonstrated with the excellent correlations between the results in SI Figure S7), but with a shorter detection time. Similar to the FO-SPR, a lower

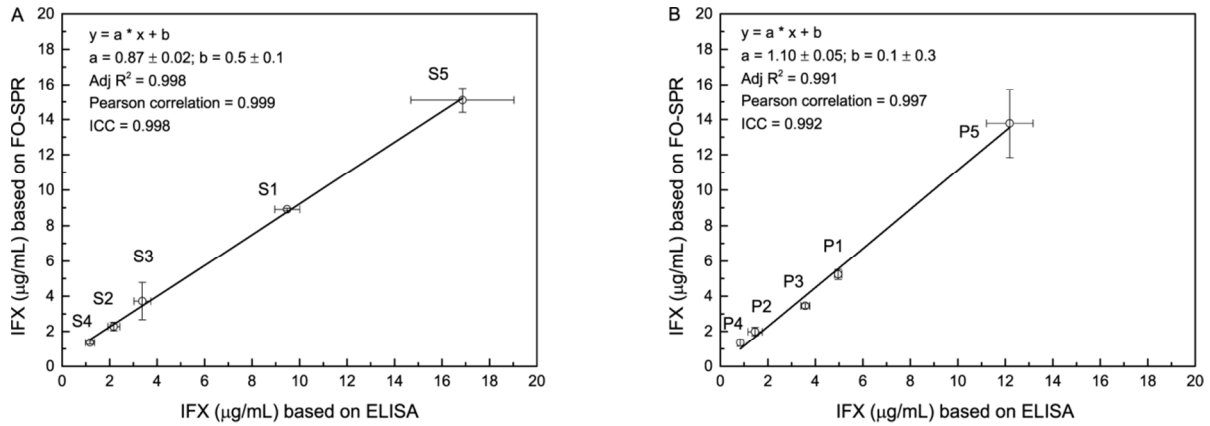


Figure 5. Evaluation of the POC FO-SPR immunoassay with serum (A) and plasma (B) samples from IBD patients. The IFX concentrations from FO-SPR platform were compared to the clinically validated ELISA. The equations displayed in the graphs represent the correlation of IFX concentrations (µg/mL) obtained based on FO-SPR and ELISA. Error bars represent standard deviation (n= 3 for both FO-SPR and ELISA).

IFX concentration was also obtained for P1 sample compared to S1 when using ELISA. The POC FO-SPR bioassay and ELISA had an excellent correlation as demonstrated with the Pearson correlation and ICC of 0.999 and 0.998, respectively for serum samples, and 0.997 and 0.992, respectively for plasma samples. The average CV of the FO-SPR from both serum and plasma samples was approximately 10%.

CONCLUSIONS

This paper describes the development and validation of a FO-SPR based immunoassay for the detection of IFX in a variety of matrices including serum, plasma, whole blood and DBS extracts. Starting from the previously published concept [3], two types of bioassays were first established for detecting IFX in 10-fold diluted blood: (i) 40-minutes bioassay and (ii) POC bioassay, with a total detection time of 13 or 10 minutes. The difference in detection time was based on whether the SPR shift (method 1) or the curve slope (method 2) from the AuNP binding step was used for generating calibration curves. The obtained LOD values were 1.12 ng/mL for the 40-minutes bioassay and 0.75 ng/mL or 0.9 ng/mL for POC bioassay based on method 1 or method 2, respectively. This demonstrated for the first time measurements of IFX directly in whole blood, thereby surpassing difficulties often linked to measurements in whole blood, such as high non-specific binding on the sensor surface and almost two times lower levels of IFX trough levels in blood compared to serum.

Next, in order to measure IFX concentrations in the clinically relevant range with the developed POC FO-SPR bioassay (i.e. 0.5 to 10 μg/mL in serum samples and 0.23 to 4.5 μg/mL in whole blood), new calibration curves were generated in 100-fold diluted serum, plasma and whole blood. The LOD values for all three matrices were below 1.5 ng/mL for both method 1 and method 2. Moreover, excellent correlations between different calibration curves indicated that the matrix effect was insignificant for the given dilution factor. Importantly, all the calibration curves were analyzed with a randomized block design. There was no statistical significance between measurements generated on two different days using two batches of independently prepared FO-SPR sensors and AuNPs. These results demonstrated that a new calibration curve is unnecessary for each batch of FO-SPR sensors and AuNPs.

The capacity of developed POC FO-SPR bioassay was further assessed using DBS samples. Following the optimized protocol for DBS sample preparation, it was calculated that the extraction efficiency of IFX was 99 %, suggesting that (i) the drying process of blood did not affect the extraction of IFX or its interaction with the antibodies used in the FO-SPR bioassay, (ii) the distribution of IFX on the DBS paper was uniform given that only part of the paper was used for extraction process and (iii) non-specific binding (due to the bursting of blood cells caused by drying of whole blood) was not interfering with the FO-SPR bioassay. The obtained LOD values were below 2 ng/mL, irrespective of whether IFX was spiked in the whole blood prior to or after the extraction. This demonstrated the potential of the POC FO-SPR bioassay to be used for testing DBS samples that are collected by finger-prick, as patients can perform sample collection by themselves at home and requirements for transportation of the DBS samples are minimal.

The final validation of the POC FO-SPR immunoassay was performed with 10 clinical samples from IFX-treated IBD patients, being five matching serum and plasma samples from the same five patients. The measured IFX concentrations were compared to the values obtained using clinically validated ELISA, revealing excellent agreement between two methods, with the Pearson correlation and ICC of 0.999 and 0.998, respectively for serum samples, and 0.997 and 0.992, respectively for plasma samples.

By comparison to the previous work, the POC FO-SPR bioassay presented in this paper has the advantages of shorter detection time (10 minutes compared to 40 minutes), lower LOD (1.05 compared to 2.2 ng/mL in 100-fold diluted serum), and comparable accuracy when testing patient samples. Importantly, it was also demonstrated that not only SPR shift but the slope of the obtained binding curves can be used for generating calibration curves. This so called method 2 in this paper allowed us to establish bioassay in complex sample matrices of only 10 minutes without compromising on the sensitivity or reproducibility of the detection as both LOD and CV values were comparable to method 1 (i.e. method based on the SPR shift measurements). In conclusion, the FO-SPR biosensor offers advantages including fully automated set-up, single sample access and rapid detection. The fact that the same platform can detect IFX in a variety of matrices, such as serum, plasma, whole blood and DBS extracts, with clinically relevant sensitivities in 10 minutes demonstrated that the FO-SPR biosensor can be used as a versatile POC diagnostic tool.

ASSOCIATED CONTENT

Supporting Information. Further experimental details and results can be found in the pdf file.

AUTHOR INFORMATION

Corresponding Author

* E-mail: jeroen.lammertyn@kuleuven.be.

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ABBREVIATIONS

AuNP, gold nanoparticle; DBS, dried blood spot; ELISA, enzyme linked immunosorbent assay; FO-SPR, fiber-optic surface plasmon resonance; IBD, inflammatory bowel disease; IFX, infliximab; POC, point-of-care.

REFERENCES

(1) Corstjens, P. L.; Fidder, H. H.; Wiesmeijer, K. C.; Claudia, J.; Rispens, T.; Wolbink, G.-J.; Hommes, D. W.; Tanke, H. J. *Analytical and bioanalytical chemistry* **2013**, *405*, 7367-7375.

(2) Van Stappen, T.; Bollen, L.; Casteele, N. V.; Papamichael, K.; Van Assche, G.; Ferrante, M.; Vermeire, S.; Gils, A. *Clinical and Translational Gastroenterology* **2016**, *7*, e206.

(3) Lu, J.; Van Stappen, T.; Spasic, D.; Delport, F.; Vermeire, S.; Gils, A.; Lammertyn, J. *Biosensors and Bioelectronics* **2016**, *79*, 173-179.

(4) Hofman, S.; Bolhuis, M. S.; Koster, R. A.; Akkerman, O. W.; van Assen, S.; Stove, C.; Alffenaar, J.-W. C. *Bioanalysis* **2015**, *7*, 481-495.

(5) de Wit, D.; den Hartigh, J.; Gelderblom, H.; Qian, Y.; den Hollander, M.; Verheul, H.; Guchelaar, H. J.; van Erp, N. P. *The Journal of Clinical Pharmacology* **2015**, *55*, 1344-1350.

(6) McDade, T. W.; Williams, S.; Snodgrass, J. J. *Demography* **2007**, *44*, 899-925.

(7) Van Stappen, T.; Lu, J.; Bloemen, M.; Geukens, N.; Spasic, D.; Delport, F.; Verbiest, T.; Lammertyn, J.; Gils, A. In *SPIE BiOS*; International Society for Optics and Photonics, 2015, pp 931705-931705-931707.

(8) Arghir, I.; Spasic, D.; Verlinden, B. E.; Delport, F.; Lammertyn, J. *Sensors and Actuators B: Chemical* **2015**, *216*, 518-526.

(9) Van Stappen, T.; Brouwers, E.; Tops, S.; Geukens, N.; Vermeire, S.; Declerck, P. J.; Gils, A. *Therapeutic drug monitoring* **2015**, *37*, 479-485.

(10) UNODC. *Guidance for the Validation of Analytical Methodology and Calibration of Equipment Used for Testing of Illicit Drugs in Seized Materials and Biological Specimens*; United Nations Office on Drugs and Crime, 2009.

(11) Barbosa, A. I.; Castanheira, A. P.; Edwards, A. D.; Reis, N. M. *Lab on a Chip* **2014**, *14*, 2918-2928.

(12) Stelter, L.; Evans, M. J.; Jungbluth, A. A.; Longo, V. A.; Zanzonico, P.; Ritter, G.; Bomalaski, J. S.; Old, L.; Larson, S. M. *Molecular imaging* **2013**, *12*, 7290.2012. 00009.

(13) Ferreon, A. C. M.; Ferreon, J. C.; Wright, P. E.; Deniz, A. A. *Nature* **2013**, *498*, 390-394.

(14) Tuaillon, E.; Mondain, A. M.; Meroueh, F.; Ottomani, L.; Picot, M. C.; Nagot, N.; Van de Perre, P.; Ducos, J. *Hepatology* **2010**, *51*, 752-758.

(15) Suyagh, M. F.; Iheagwaram, G.; Kole, P. L.; Millership, J.; Collier, P.; Halliday, H.; McElnay, J. C. *Analytical and Bioanalytical Chemistry* **2010**, *397*, 687-693.

(16) Vu, D. H.; Koster, R. A.; Alffenaar, J. W. C.; Brouwers, J. R. B. J.; Uges, D. R. A. *Journal of Chromatography B* **2011**, *879*, 1063-1070.