Self-calibrating on-a-chip LSPR sensing for quantitative and multiplexed detection of cancer markers in human serum

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Table of Contents Graphic

168x61mm (150 x 150 DPI)
Self-calibrating on-a-chip LSPR sensing for quantitative and multiplexed detection of cancer markers in human serum

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Abstract - The need for point-of-care (POC) devices capable of early detecting diseases and monitoring their status, out of a lab environment, has stimulated the development of compact biosensing configurations. While Localized Surface Plasmon Resonance (LSPR) sensing integrated into a state-of-the-art microfluidic chip stands as a promising approach to meet this demand, its implementation into an operating sensing platform capable to detect quantitatively a set of molecular biomarkers in an unknown biological sample is only at its infancy. Here, we present an on-chip LSPR chip capable to perform automatic, quantitative and multiplexed, screening of biomarkers. We demonstrate its versatility by programming it to detect and quantify in human serum four relevant human serum protein markers associated with breast-cancer.

Early diagnosis and accurate monitoring of disease progression is required to determine an optimum treatment and increase the recovery rate of patients. The complex nature of biological processes and pathways leads to significant changes in the levels of multiple molecular markers in patient’s blood over the course of a disease¹⁷–²². Therefore, it is highly relevant to be able to track simultaneously the levels of a set of these markers, in order to spot the actual disease status. Enzyme-linked immunosorbent assay, ELISA is one of the most commonly used detection technique in clinics and research laboratories. Its reliability, low limit of detection and commercial availability are the biggest advantages. However, the bulky well-plates and readers, long assay times and the large reagent volumes are not compatible with on-site, quick and multiplexed measurements. The ultimate goal of developing point-of-care (POC) devices that allow for on-site, multiplexed measurement of analytes in a fast and cost-effective manner has motivated the development of a variety of biosensors based on different transduction mechanisms⁸–¹⁰. Among them, optical sensors are particularly attractive owing to their fast response and compatibility with miniaturization and parallel sensing⁸–¹¹. Surface Plasmon Resonance (SPR) sensing is one of the well-developed and commercially available optical sensing schemes.

A competitive approach to optical sensing relies on Localized Surface Plasmon Resonance (LSPR) supported by noble metal nanoparticles. Unlike SPR-sensing based on flat metal films, LSPR-sensing enables engineering the sensing volume down to the subwavelength (molecular) scale and benefits from direct coupling with propagating light. LSPR-based biosensing has shown to be a powerful approach to compact and simple platforms, especially attractive for POC applications⁹,¹¹–¹⁷. Yet, despite its great potential, its implementation into operating sensing devices capable to quantitatively assess the analyte concentration from unknown biological samples, remains little advanced¹⁸–¹⁹. This is in part due to the complexity of implementing a working platform, where several disciplines like physics, surface chemistry, fluids and electronics have to be optimally combined. Furthermore, while most efforts have so far focused on the detection of single analytes, reliable detection and monitoring of diseases generally requires multiplexed detection of several biomarkers. To this end, earlier works proposed employing colloidal metallic nanoparticles with different LSPR frequencies, each functionalized with a different receptor²⁰. While promising, this solution-based approach currently faces several drawbacks including aggregation of nanoparticles and optical signal fluctuations due to multiple washing steps. Another related configuration for multiplexing consists in immobilizing on a substrate antibody-functionalized gold nanorods of different aspect ratios²¹ that exhibit different optical properties allowing the selection of multiple working wavelengths. Chen et al. developed a multiplex serum cytokine immunoassay using nanoplasmonic biosensor microarrays. In this
configuration, the solution based gold nanorods were immobilized on a substrate using a microfluidic channel network which was then removed and replaced by another microfluidic network used for the sensing assay. To date, none of the proposed LSPR-based schemes has enabled multiplexed and quantitative detection of several analytes in a biological sample.

As a first step towards this goal, we recently presented a strategy that combines top-down engineered gold nanoparticle arrays with state-of-the-art microfluidics comprising micromechanical valves. This unique combination, which provides a controlled environment to the sensors, accurate delivery of reagents and sample as well as automated assay operation, was successfully used to detect protein cancer markers in human serum with high sensitivity and specificity. As a proof of principle, detection of prostate specific antigen (PSA) and alpha-fetoprotein (AFP) in a clinically relevant level range were successively obtained.

Here, by leveraging on the developed toolbox, we report on the first implementation of quantitative and multiplexed LSPR sensing on-a-chip. Our LSPr chip enables us to perform simultaneously self-calibrating, automated and multiplexed real-time detection of four breast cancer protein markers in human serum. These sensing performances combined with the long shelf-life of the chips, brings the LSPR based sensing one step closer to real-life operating POC devices.

**Clinical needs** – Clinical studies show that accurate detection of serum protein markers for breast cancer are pivotal to treatment monitoring towards a better prognosis. The most common markers are CA (cancer antigen) 15-3 and CEA (carcinoembryonic antigen) as well as high serum ErbB2 (HER-2/Neu) concentrations are shown to be of use to monitor the response to specific treatment types. In addition to these antigens, the CA 125, which is a serum marker for ovarian cancer and some other diseases, is also shown to be a predictive marker of metastasis in breast cancer patients. More serum protein markers are relevant to breast cancer; extensive reviews can be found in the literature. Since tracking the level of a single marker alone may not be sufficiently conclusive in most cases, one needs to be able to monitor multiple markers in parallel. In our multiplexed measurements, we have focused on the four aforementioned molecules to demonstrate that our platform enables us to reliably quantify the concentrations of four relevant molecules with high specificity and reduced cross-reactivity.

**Description of the sensing chip** – To meet all requirements for quantitative multiplexed sensing, our chip design makes use of orthogonally crossing channels compartmented by micro-mechanical valves. The eight microfluidic channels intersect with four orthogonal channels and each intersection area contains an array of gold nanorods sensors (Figure 1 and Figure 2). Eventually, the density of sensing sites is 32/mm², enabling time-shared parallel detection over different positions by using a galvanometric mirror in a transmission measurement setup. The dimensions and the periodicity of the gold nanorods in this work are similar to some previous works. Gold nanorods are 160 nm x 80 nm with thickness of 50 nm and arranged in a square lattice of 400 nm pitch. This design was selected to minimize near and far field coupling effects and to lead to a strong resonance in the extinction signal. Figure 1a displays the chip outline where blue lines depict the flow network and red lines the valve-actuation architecture for flow control. E-beam lithography with negative resists combined with reactive ion etching were used to fabricate gold nanorod arrays on a glass substrate (Figure 1b). Subsequently, the surface of the nanorods was coated with a self-assembled monolayer (SAM) of MUA (mercaptoundecanoic acid) for further modification aimed at specific analyte binding. Finally, we aligned and assembled the sensor substrate with microfluidic channels, fabricated by multilayer soft lithography, in such a way the nanorod arrays sit at the intersection points of orthogonal channels (Figure 1a). By regulating the pressure inside the control lines, the flow on the chip can be actuated to address specific detection sites (Figure 2c). It is noteworthy mentioning that once assembled, the chip can be stored at room temperature, in dark, up to 3 months without any significant alteration of its performance. Figure S1 in Supporting Information shows the antibody binding performance of 1-week old and 3-months old chips, as well as associated calibration curves obtained by sandwich assay for CA15-3 protein.

For the biomarker detection on chip, the micromechanical valves connected to electronic valves are controlled by a home-made Matlab GUI (Figure S2) in order to run an automated sandwich assay, where a capture antibody is first immobilized on the sensor in order to specifically capture the protein of interest and, when needed, the LSPR shift is subsequently amplified with an amplification antibody (Figure 1c and d). The LSPR centroid red-shift at the detection sites due to bioanalyte binding is tracked in real-time with a home-made transmission spectroscopy setup controlled by a Labview interface. Figure 1e shows the real-time measurement of the successive assay steps for obtaining CA15-3 calibration curve, involving only 25% of the total sensing sites available on the chip. The EDC/NHS activation of the carboxyl groups of the SAM is followed by the capture antibody immobilization on 8 sensors, onto which 8 different concentrations of the marker are introduced. The final step (zoomed in inset) shows the amplification antibody binding at the 8 different detection sites, resolving the different concentrations of the marker (Details in Supporting information).

The inlets and outlets of the chip are designed such that the antigen cocktail samples and the capture and amplification antibody solutions are connected to the chip through tygon tubing and they can be flowed whenever needed without any further manual manipulation. In our set-up, electronic valves that control the micromechanical valves through a home-made matlab GUI software and embedded scripts
(Figure S2) enables full automation of the measurement protocol. Controlled flow of different solutions in specific directions and without cross contamination is shown in Figure 2a, where the main three flow modes of the microfluidic operation are captured using food colorant (Also see Supporting Video 1). This way, every detection site can be treated simultaneously with the same reagent/solution through a common inlet (Figure 2a, mode i). Additionally, different solutions can be delivered to the detection sites through the orthogonal channels in different directions as shown in Figure 2a, mode ii and Figure 2a, mode iii. These different flow modes, together with the low dead volumes and absence of cross-talk between channels, are crucial for reliable multiplexed measurements.

![Microfluidic LSPR sensing chip diagram](image)

Figure 1. Description of the microfluidic LSPR sensing chip. (a) Design of the microfluidic chip where the blue lines represent the flow network and the red lines represent the valve control network of the multilayer PDMS chip. The yellow lines inside the blue channels locate the gold nanorod array. (b) Scanning electron micrograph of the gold nanorod arrays on glass. (c) Sandwich assay sensing scheme, where the capture antibody (cAb) is immobilized on the sensor after being coated with MUA (Step1), the target biomarker is captured by the cAb (Step2), and the amplification antibody (aAb) is subsequently bound to the marker, as an amplification step to enhance the LSPR shift (Step3). (d) Real-time monitoring of the LSPR centroid shift for different concentrations of CA15-3 (on-a-chip calibration curve).
In the specific multiplexed sensing assay, the common treatment of all sensor sites (mode i) is used to activate the gold sensor surface with EDC/NHS chemistry and for common blocking and washing steps. Mode iii is employed to immobilize the 4 different capture antibodies on the four sensor arrays (Figure 2b and c, Step 1). Six of the eight parallel channels are used, utilizing mode ii, to deliver to the sensors the cocktail of proteins with different concentrations and obtain the calibration curves of the four proteins. The last two channels are simultaneously used for the unknown sample replicas to be quantified (Figure 2b and c, Step 2). Finally, whenever necessary, the amplification antibodies are flowed through to amplify resonance shift (Figure 2b and c, Step 3). More details on the surface chemistry and marker detection protocol can be found in Supporting Information. The contribution of the flow control by the micromechanical valves to both the sensor functionalization and the sensing measurements, is illustrated by the cross-section view in Figure 2c.

Figure 2. Description of the chip operation protocol for the multiplexed detection of four protein cancer markers. (a) Pictures of the microfluidic channel flow layer at different instants of the sensing protocol. For sake of clarity, food dyes were used. The 8 channels can be either fed by a common inlet (mode i) or addressed individually (mode ii). In mode iii, the 4 orthogonal channels are addressed separately and flow can be adjusted to be in two opposite directions. (b) Steps of the multiplexed sensing assay in which the capture antibodies of different markers are first immobilized on the surface (Step 1), then the marker cocktails with different concentrations [M]i are flown through individual channels (Step 2) and finally the amplification antibody of respective markers are flown through the orthogonal channels (Step 3). (c) Cross section of the chip at the position marked by the black rectangle on (b) illustrating the flow directions and the valve operation.

With this novel LSPR chip design, the high density of addressable sensing sites increases the throughput and a proper configuration of the assay via the flow step sequence enables the chip to perform self-calibrations. Remarkably, the on-chip calibration for a given analyte can be simultaneously recorded while measuring the actual unknown biological sample, improving the reliability of the quantification. Equally important, the orthogonal channels allow the antibodies to be immobilized exclusively on the sensing areas, thus significantly reducing parasitic effects of analyte depletion (due to non-specific interactions), and allowing more freedom in passivation strategies. Finally, the whole biosensing assay can be fully programmed and automatized for a wider range of applications based on label-free operation (for instance, affinity screening in pharmaceutical industry). All these advanced features of microfluidic platform combined with the real-time LSPR centroid shift tracking, enables a reliable, multipurpose platform.
Optimization of antibody concentrations - To ensure the quality of the on-chip sandwich assays, we have first optimized the capture and amplification antibody concentrations and determined the analytical performances of each of the 4 molecules, individually.

In order to determine the optimum concentrations of each of the capture antibodies, we first immobilized 4 different concentrations of capture antibody for the marker of interest and then flowed 8 different marker concentrations, followed by the amplification antibody solution at constant concentration. From these collection of data, all obtained from a single chip, one can plot four different calibration curves, one for each of the capture antibody concentrations. The best capture antibody concentration was selected accounting for the high slope, low unspecific signal and high sensitivity. To compensate for the reduced dynamical range, one can use sample dilution. Alternatively, antibody concentration and assay conditions can be optimized to obtain larger dynamic ranges. Following a similar approach, on another chip, we optimized the amplification antibody concentrations, immobilizing the optimum solution of capture antibody on the sensors and running the calibration curve measurements with 4 varying amplification antibody concentrations this time. Figure 3a and 3b, shows the optimization measurements for CA 15-3.

Following this protocol, we determined the optimum concentrations of all the antibody pairs used for further sensing applications (See Supporting Information and Figure S3). As a result, the individual calibration curves for the 4 protein markers to be detected, by optimized antibody concentrations are obtained (Figure 3 c-f). The dynamic ranges and the limit of detections of these individual marker measurements can be found in Table 1. For ErbB2 protein, the direct protein detection without amplification antibody signal was sufficient to obtain a sensitive enough calibration curve. This effect can be due to the possible higher affinity constant of the selected antibody for ErbB2 compared to the other proteins or effects related to the 3D structure of the protein or steric hindrances when the antigen is immobilized on the sensor surface.

In order to have a clear comparison between different measurements and different markers, the normalized calibration curve data is presented in Figure 3 (See Supporting information for the raw LSPR shifts (Figure S4)). The error bars on the calibration curve data in Figure 3 represent the variation between replicas from different chips for CA 15-3 and CA 125 and on the same chip for CEA and ErbB2. The relative standard deviation between replicas in the dynamic range is ~14% for interchip measurements and ~19% for intrachip measurements, suggesting that the reproducibility on different chips is as high as on the same chip. Owing to the self-calibration capacity of the platform, the sample quantification performance of the platform is independent of such small variations.

The detection sensitivity extracted from the calibration curves for CA 15-3, Erb2 and CEA are compatible with clinical cut-off values28,29,31,36. For CA 125, the not so good limit of detection is attributed to the low quality of available antibodies.

![Figure 3. Assay optimization and individual calibration curves. (a) and (b) shows the antibody optimization experiments for CA 15-3. (c-f) are the individual calibration curves for the four biomarkers obtained for optimized antibody concentrations. Error bars reflect the deviation between replicas on different chips for (c) and (e), and on the same chip for (d) and (f).](image)

**Cross-Reactivity** - Nonspecific antibody-protein cross-reaction results in inaccuracy and unreliable sensor response, interfering with the purpose of multiplexing60,61. In order to verify the specificity of the platform, we conducted a cross-reactivity control experiment (Figure 4a), where we immobilized the capture antibodies against CA 15-3, CA125, CEA and Erb2 on four sensor arrays (flow mode iii) and we
flowed high concentrations of the four proteins individually (flow mode ii). Concentrations of proteins were selected to be the maximum ones of the dynamic ranges of their respective calibration curves. LSPR shifts obtained after sandwich formation for CA 15-3, CA125 and CEA channels and during the sample flow for ErbB2 channels are presented in Figure 4b. No cross-reactivity was observed between different species despite the high concentrations of proteins used. As an example, the calibration curve of CA15-3 obtained in the presence of ErbB2 molecule is presented in Figure S5. Our data show that the platform ensures limited cross-contamination between the channels and the antibody selections prevent any significant cross-reactivity.

![Diagram of cross-reactivity experiments](https://example.com/diagram.png)

**Figure 4. Cross-reactivity experiments.** (a) Sketch of the experimental steps (For sake of clarity, the control layer of the chip is not shown). Capture antibodies are immobilized and the proteins are flowed separately in different channels. Four replicas of the controls with no proteins is flowed also to check for channel-to-channel variation of the signals. Every intersection of the orthogonal flow channels is a sensing region. The sensing regions corresponding to each matching antibody-protein pair is marked with a star. (b) Corresponding LSPR shifts on the sensors. Shifts for the control channels are merged and the error bar is associated to the standard deviation of four replicas for the control measurement with each antibody pair. The protein concentrations are 18U/ml, 9000U/ml, 3200ng/ml and 600ng/ml for CA 15-3, CA 125, CEA and ErbB2 respectively.

**Multiplexed measurement in PBS with 1% BSA buffer** - As a proof of principle experiment of multiplexed detection of the 4 biomarkers, we performed calibration curve measurements in 10mM PBS buffer with 1% BSA (bovine serum albumin) as a blocking agent. Each of the four capture antibodies was immobilized on a different sensor array (utilizing mode iii and step 1 described in Figure 2) and 8 cocktails of proteins with varying concentrations were flowed into the chip (utilizing mode ii and step 2 described in Figure 2) before introducing the amplification antibodies (Figure 2b, step3). Normalized calibration curves obtained for each protein marker are presented in Figure 5. Multiplexed measurements exhibit similar response compared to individually obtained calibration curves (dashed lines in Figure 5), showing no cross-reactivity between the four proteins. A detailed comparison of the sensitivity (half maximal effective concentration, EC50), limit of detection (LOD at EC10), and the dynamic ranges of the curves (EC20-EC80) is presented in Table 1.

**Multiplexed detection in human serum** – Following the multiplexed measurement in buffer, we demonstrate here multiplexed detection in 100% human serum. Figure 6a demonstrates the calibration curves obtained simultaneously from a single chip. Table 1 summarizes the LOD, sensitivity and dynamic range of the four markers measured in human serum. Due to the complex matrix, the curves are shifted towards higher concentrations compared to the measurements in PBS-BSA(1%) (dashed lines in Figure 6a) and therefore the LOD and EC50 values are slightly higher. Measurements were repeated 3 times to demonstrate their reproducibility. Data, presented in Figure S6, exhibit very similar characteristics, suggesting high interchip reproducibility, especially in the sandwich assays for CA 15-3, CEA and CA125. The calibration curves for ErbB2 show larger dispersion, mostly because, for this molecule, no amplification antibody was used. Therefore for this application, the sandwich assay approach with detection antibodies provided more reproducible curves in complex media.
As a final step, towards real multiplexed sensing experiment on an unknown clinical sample, we demonstrate multiplexed sample recovery. To this end, we prepared six cocktails with varying biomarker concentrations for calibration curve. Then for recovering purpose, we spiked two samples with a mixture of targeted markers at different levels in human serum to mimic an unknown clinical sample. The spiked sample concentrations were interpolated from the calibration curves that were simultaneously acquired. Figure 6b shows an example recovery measurement in whole human serum for four molecules obtained for various replicas. The recovery rates (R) are listed in the table. Two of the sample concentrations, being CA15-3 and CA 125, were found to be significantly different but not incoherent (120% < R ≤ 130%). The recovery value for CEA was underestimated (R < 80%). ErbB2 concentration in the spiked sample is below the linear range of the calibration curve, in order to demonstrate the sensing performance below the LOD. The prepared and quantified ErbB2 concentrations were 15 ng/ml and 20±20.2 ng/ml respectively. The uncertainties on the recovery are mostly attributed to spiking errors during sample preparation.

While our results are satisfying, this remains as proof of principle and there is quite some room left for boosting the platform performances. At that stage, the LOD levels for CA125 and CEA are higher than clinical cut-off values. The significant LOD difference between the different markers is attributed to the uneven performance of the different commercially available antibody pairs as discussed by Volpetti et al. Sensitivity and recovery rates could be further improved by i) optimizing the assay buffer for human serum, ii) managing better antibody pairs that will result in better LOD and sensitivity, iii) changing the assay type (competitive assay, etc.) or iv) engineering nanoparticle geometry.

Figure 5: Multiplexed detection of four molecules in PBS. Solid lines are the four-parameter logistic equation fit to the data. The dashed lines are the fit to the individually obtained calibration curves from Figure 3.

Finally, in order to benchmark our technology with an established standard, we performed ELISA measurements on all four markers, using the same antibody pairs. We first optimized the capture and detection antibody concentrations for ELISA. For the sandwich step of ELISA, we selected a commercial polyclonal antibody for ErbB2. All the rest of the antibody pairs are used in the same way as in the on-chip measurements. Calibration curves obtained with ELISA are presented in Supporting Information, together with the details of the whole ELISA protocol. From this direct comparison, we show that the LOD for CA15-3, CA125 and ErbB2 molecules are lower for on-chip measurements, outperforming the corresponding ELISA measurements. Another

<table>
<thead>
<tr>
<th>Biomarker</th>
<th>Cut-off concentration</th>
<th>Measurement</th>
<th>LOD</th>
<th>Sensitivity</th>
<th>Dynamic Range</th>
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<tr>
<td>CA 15-3</td>
<td>25-40 U/ml</td>
<td>Individual</td>
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<td>2.0 U/ml</td>
<td>0.30-8.9 U/ml</td>
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<td></td>
<td></td>
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<td></td>
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<td>12.522 kU/ml</td>
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<td>CEA</td>
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<td>14.7 ng/ml</td>
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<td>ErbB2</td>
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<td>99.8 ng/ml</td>
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Table 1. The analytical parameters of the assays on chip summarized for individual marker detection (Figure 3c-f), multiplexed detection in PBS buffer (Figure 5) and multiplexed detection in human serum (Figure 6a).
advantage our platform holds is its shorter total assay time, which in the commercialized kits, reported as 5 hours for ELISA, where the capture antibody is already immobilized inside the wells. After the capture antibody is immobilized on our sensors, our assay time is maximum 2.5 hours which can be seen in Figure 1e. The detection antibody step is now set at 1 hour but can be easily reduced to 15-20 minutes (as can be seen from the time traces) without altering the LOD levels significantly. Finally, the reagent volume used for the flowing and washing steps on-chip is 10 to 10000 folds smaller than the reagent volume used for ELISA measurements, for sensing and washing steps respectively. With the constant flow rate on-chip, the reagent consumption is less than 50 µl/hour.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Sample prepared</th>
<th>Quantified</th>
<th>Recovery rate</th>
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</thead>
<tbody>
<tr>
<td>CA 15-3</td>
<td>14.5 U/ml</td>
<td>18.3 ± 1.2 U/ml</td>
<td>126%</td>
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<tr>
<td>CA 125</td>
<td>1.9 kU/ml</td>
<td>2.30 ± 0.07 kU/ml</td>
<td>121%</td>
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<td>CEA</td>
<td>250 ng/ml</td>
<td>193 ± 52 ng/ml</td>
<td>77.2%</td>
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<tr>
<td>ErbB2</td>
<td>15 ng/ml</td>
<td>&lt;LOD</td>
<td>-</td>
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</table>

Figure 6. Sample quantification in human serum. (a) Calibration curves simultaneously obtained in 100% human serum (solid lines), and in PBS buffer from Figure 5 (dashed lines). (b) Sample recovery in 100% human serum. The concentrations of each marker is quantified by interpolating the measured LSPR shifts from the simultaneously acquired calibration curves. The table summarizes the recovery for each of the markers.

Additionally, a summary of comparison between on-chip multiplexed LSPR, ELISA and SPR methods can be found in supporting information (Table S-1).

**Conclusions** - In this work, we demonstrated how LSPR sensing integrated with state-of-the-art microfluidics enables to achieve quantitative multiplexed detection of 4 breast cancer protein markers in human serum. This demonstration stands as an important milestone in the field of LSPR sensing and brings us closer to stand alone and compact automated point of care platforms with a suitable balance between compactness, reliability and sensitivity. In the presented work, miniaturization efforts have been exclusively focused on the sensing chip. Further integration on both the optical measurement and valve controlling set-ups would be required for point of care applications. Additionally, the sensor fabrication costs can be reduced by using mass fabrication methods such as nanoimprintertm, hole-mask colloidal lithography (HCL) both compatible with microfluidic platforms. The next steps for improvement should involve validation of the platform on clinical samples including direct comparison with gold standard techniques. In order to overcome the complex media effects of clinical samples, either sample dilution or standard addition technique can be implemented. While tested here in the context of medical diagnostics, the proposed scheme is compatible with a wide range of applications. For instance, in the context of drug discovery, it would potentially allow high throughput screening combining very low reagent consumption with access to real-time binding kinetics.
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