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Magnetically-actuated, bead-enhanced silicon photonic immunosensor

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Abstract

Magnetic actuation has been introduced to an optical immunosensor technology resulting in improvements in both rapidity and limit of detection for an assay quantitating low concentrations of a representative protein biomarker. For purposes of demonstration, an assay was designed for monocyte chemotactic protein 1 (MCP-1), a small cytokine which regulates migration and infiltration of monocytes and macrophages, and is an emerging biomarker for several diseases. The immunosensor is based on arrays of highly multiplexed silicon photonic microring resonators. A one-step sandwich immunoassay was performed and the signal was further enhanced through a tertiary recognition event between biotinylated tracer antibodies and streptavidin-coated magnetic beads. By integrating a magnet under the sensor chip, magnetic beads were rapidly directed towards the sensor surface resulting in improved assay performance metrics. Notably, the time required in the bead binding step was reduced by a factor of 11 (4 vs 45 min), leading to an overall decrease in assay time from 73 min to 32 min. The magnetically-actuated assay also lowered the limit of detection (LOD) for MCP-1 from 124 pg mL⁻¹ down to 57 pg mL⁻¹. In sum, the addition of magnetic actuation into bead-enhanced sandwich assays on a silicon photonic biosensor platform might facilitate improved detection of biomarkers in point-of-care diagnostics settings.

Introduction

The detection of protein biomarkers continues to grow in importance as these biomolecules are increasingly used as indicators of disease state. In addition to having sufficient sensitivity and appropriate limits of detection, diagnostic technologies must also provide results in timeframe consistent with the requirements of the setting. An increasing number of reports highlight the use of micro- and nanoscale sensors to achieve extraordinary sensitivity (pg mL⁻¹ level).^{1–4} However, these reduced sensor sizes are accompanied by challenges associated with slow diffusion to the sensor surface, resulting in long assay times to achieve the ultimate detection limits.^{5, 6} Lengthy analysis times often represent a hindrance to the translation of many promising biomarker detection technologies to point-of-care applications. For example, many well-established and commercialized biomarker detection methods, including plate-based ELISAs, Luminex, and the more recently developed ultrasensitive Erenna assay system, each have total assay times of several hours or longer.

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Therefore, there still exists a need for new technologies that can provide relevant analytical metrics but with significantly reduced time-to-result.

Over the past several years, our group has developed a biosensing technology based on silicon photonic microring resonators.^{7, 8} This technology leverages semiconductor fabrication methods to create arrays of sensors (typically 32 or 128 sensor elements) that can be used for multiplexed biomarker detection. Each individual microring supports optical resonances, and the spectral position of these resonances is sensitive to changes in the local refractive index. When functionalized with an analyte-specific capture agent, binding of the target biomolecule at the microring surface leads to a resonance shift that can be monitored as a function of time. Both the rate and absolute magnitude of the resonance shift can then be utilized to determine the concentration of biomarkers within the sample.

We previously demonstrated the general utility of these sensors for the detection of a range of biomolecular targets, including proteins,⁹ nucleic acids,¹⁰ whole virus particles,¹¹ and biotoxins,¹² in both single- and multiplex formats. Additionally, we have shown the ability to quantitate biomarker levels from within complex sample matrices such as crude plant extracts,¹¹ cell culture media,^{13, 14} and human body fluids, including serum and plasma,¹⁵ and cerebrospinal fluid.¹⁶ Notably, for detection on complex sample matrices, assay specificity is often increased through the use of sandwich assay formats, and the addition of the tracer antibody affords additional methods for improving assay performance. Specifically, in several recent studies we have exploited the sandwich assay format to reduce limits of detection by incorporating enzymes or beads as labels.^{15, 16}

Of particular relevance to this manuscript, the bead based enhancement strategy demonstrated both an improvement in limit of detection and an increase in dynamic range, due to the incorporation of a 3-step assay format. However, multi-step assay that included the relatively slow diffusion of beads to the microring surface necessitated an assay time of ~70 minutes. Given that the beads utilized in that assay have magnetic cores, and inspired by related studies on a related surface plasmon resonance platforms,^{17, 18} we reasoned that the total assay time could be significantly decreased by using a magnetic field (magnetic actuation) to draw the beads to the surface. This would overcome diffusion-based limitations and minimize the time period needed to achieve the maximum resonance shift.

Monocyte chemotactic protein 1 (MCP-1; also known as CCL2) was chosen as a model biomarker for demonstrating the rapid detection of a clinically-relevant target. MCP-1 is a cytokine that has been highlighted as a potential marker of sepsis.¹⁹ Given its acute nature and rapid progression, sepsis is a prime example of a clinical presentation that would benefit from rapid diagnostics as the rate of survival is strongly correlated with time to therapeutic intervention.²⁰ Considering just the detection of MCP-1, as one of several potential biomarkers for sepsis, commercial ELISA kits feature total assay times of 3+ hours, highlighting the need for more rapid diagnostic approaches.

Although magnetic fields have been utilized to enhance the performance of other sensing technologies, this is the first time that magnetic actuation has been coupled with a microcavity optical resonator-based detection strategy to directly attract the magnetic beads

to the sensing platform, minimizing the assay time. In this work, we incorporate a magnetic field to actively direct beads down towards the microring sensor surface in a sandwich immunoassay for MCP-1. We directly compare the use of a magnetic field versus passive diffusion of the magnetic beads in a standard flow configuration. We observed that the limit of detection was more than two times improved when the magnet was employed (57 vs. 124 pg mL⁻¹). We also find that the incorporation of the magnet allowed reduction of the time required in the bead binding step by a factor of 11 (4 vs 45 min). As a consequence, the total assay time was shortened from 73 min to 32 min. Importantly, this factor of two reduction in assay time could be particularly significant when using inflammatory-based diagnostics for urgent medical conditions such as sepsis, where patient survival rapidly decreases with every hour delay in therapeutic intervention.²¹ These assay improvements, coupled with the analytical versatility and multiplexing capability of the silicon photonic detection platform, position it as an attractive technology for point-of-care biomarker detection.

Materials and Methods

Materials

Anti-Human MCP-1 (anti-CCL2; catalog number 14-7099), biotinylated anti-MCP-1 (anti-CCL2; catalog number 13-7096), and recombinant human protein MCP-1 (CCL2; catalog number 14-8398) were purchased from eBioscience, San Diego, CA. Mouse IgG (catalog number ab37355) was purchased from abcam, Cambridge, MA. The assay running buffer (PBST) was made by reconstituting Dulbecco's Phosphate Buffered Saline (Sigma, catalog number D5773-50L) to include 0.05% Tween20 (Sigma, catalog number P9416-100mL). Starting Block (catalog number PI37538) was purchased from Fisher. Glycine (catalog number 12007-0010) was purchased from Acros (New Jersey). Streptavidin coated magnetic beads with ~200 nm diameter (catalog number 0.22) were purchased from Ademtech (Pessac, France; distributed by Accurate Chemical & Scientific Corp, Westbury, N.Y.). Bis[sulfosuccinimidyl] suberate (BS3; catalog number 21585) and 3-aminopropyltriethoxysilane (APTES; catalog number 80370) were purchased from Thermo Scientific. Small neodymium magnets measuring 1/8" × 1/8" × 1/16" and magnetized through thickness were purchased from K & J Magnetics, Pipersville, PA (catalog number B221). All other chemicals were purchased from Sigma and used as purchased.

Sensor fabrication

Sensor chips (6×6 mm) and read out instrumentation were obtained from Genalyte, Inc. (San Diego, CA) and details of their operation in sensing experiments have been previously described.^{7, 8} Briefly, arrays of 32-individually addressable microring sensors (30 μm diameter) were fabricated into the top layer of silicon-on-insulator at a commercial-scale silicon foundry (IMEC). Proximal to each microring resonator is a linear interrogation waveguide, through which the optical properties of the microring can be probed by coupling light through input and output diffractive grating couplers. Post fabrication, the entire chip surface is coated with a perfluoropolymer and then photolithography and reactive ion etching were used to open annular openings over 24 of the microrings. These microrings

come into contact with the flowing solution during a detection experiments and the remaining 8 occluded rings serve as thermal controls.

In a sensing experiment, sensor chips are loaded into a previously described fluidic cartridge. Solutions are then flowed directly across the surface through microfluidic channels defined through a laser cut Mylar gasket (0.007" thick) under the control of a syringe pump. The assembly was loaded into the instrumentation and light was coupled into the sensor grating coupler from a tunable external cavity laser. Each sensor was monitored in a serial fashion with resonance wavelengths monitored in real-time as a dip in transmittance as the laser wavelength is scanned across a suitable spectral range.

Sensor functionalization

Prior to functionalization, sensor chips were briefly immersed in acetone (2 min) with gentle swirling to remove a protective photoresist coating on the chip surface. Chips were then submerged in 5% APTES in acetone (4 min) followed by submersion in acetone (2 min) with gentle swirling and submersion in isopropanol (2 min) with gentle swirling. Chips were then dipped briefly in water and blown dry with compressed air or nitrogen. Following this 50 μL of a solution of BS3 (2.86 g L^{-1} , in 2 mM acetic acid, a water-soluble homobifunctional crosslinker that reacts with amines) was spotted by hand onto the chip surface and allowed to sit for 3 minutes. The chip was then blown dry.

To functionalize with a capture agent solutions, microrings were spotted with either the capture antibody (Anti-Human MCP-1, 0.3 mg mL^{-1} , in 10 mM PBS with 5% glycerol) or with an off-target control (Mouse IgG, 0.3 mg mL^{-1} , in 10 mM PBS with 5% glycerol). Spots of 0.6 μL were placed over the desired groups of microrings with the aid of a stereoscope and allowed to incubate in a humidity chamber for at least one hour. The surface of the chip was then rinsed with starting block and stored submerged in a 1 mL aliquot of starting block at 4 $^{\circ}\text{C}$ until use.

One-step-sandwich pre-formation

The antigen-tracer antibody complex was formed off chip prior to introduction to the sensor by incubating samples (0.4 mL) having various MCP-1 concentrations with biotinylated tracer antibody (3.22 μL , stock concentration: 500 $\mu\text{g mL}^{-1}$) for 15 minutes prior to microring analysis.

Magnetic bead exchange procedure

We have found that using freshly prepared magnetic bead solutions with carefully controlled bead concentrations is crucial for accurate quantitation. Prior to a detection experiment, an aliquot of 10 μL of the magnetic bead stock solution was placed in a 1.5 mL microcentrifuge tube. The magnetic beads were separated using a magnet and washed once with 100 μL of PBST buffer. The beads were resuspended in 750 μL of PBST buffer and absorbance at 286 nm was measured on a Nanodrop instrument. PBST buffer was then added to the bead solution to adjust the absorbance to 0.1. The bead solution was freshly prepared immediately before each detection experiment.

Removable magnet array configuration

For the magnetic field actuation, a custom chip holder was designed to have a removable tab attached to a plastic handle that facilitated insertion and removal of the magnetic field. We empirically found that an array of nine neodymium magnets, (1/8 inch diameter × 1/16 inch deep round) arranged in a 3×3 array with alternating north and south poles facing upwards provided a more homogeneous magnetic field for equivalent bead pull down across the array, compared to a single magnet of equal physical dimensions.

MCP-1 assay without magnetic enhancement

In the case of the assay without the magnetic actuation, the MCP-1 assay was conducted as follows. First, the functionalized sensor chip was loaded into the fluidic cartridge with a gasket with containing two side-by-side channels. PBST was flowed across the chip (2 min) followed by glycine (3 min), and then PBST (10 min). The one-step sandwich solution was then flowed (10 min) across the chip with different MCP-1 concentrations in each channel followed by PBST (2 min). The freshly prepared magnetic bead solution was then flowed across the chip for 45 min in the absence of a magnetic field. Finally, PBST was flowed for 1 min to allow determination of the final net shift for the bead enhancement step.

MCP-1 assay with magnetic enhancement

The magnetically actuated MCP-1 assay was conducted identically to that described above up until the point that the magnetic beads were introduced. After the introduction of the bead solution, the magnet array was then physically cycled in and out of the cartridge in 10 s intervals for a total of 3 min. Finally, PBST was flowed for 1 min to allow determination of the final net shift for the bead enhancement step.

Data analysis

Shifts in the resonance wavelength were determined by measuring the difference in resonance wavelength of the anti-MCP-1-functionalized microrings before the introduction of beads and then after the solution was returned to buffer following signal enhancement. The shift from off-target mouse IgG-functionalized control microrings was also subtracted to account for non-specific adsorption.

Standard curves were obtained, using GraphPad Prism 5.00, plotting the total shift data in respect to the MCP-1 concentration and fitting the points to the logistic four-parameter equation,

$$f(c) = \frac{A_1 - A_2}{1 + 10^{(C_0 - X) * p}} + A_2$$

where A_1 is the maximum shift variation corresponding to the maximum amount of target, A_2 is the minimum shift variation corresponding to the minimum amount of target, c_0 is the center of the fit, and p is the slope factor. Limit of detection (LOD) was estimated as the concentration providing the average of the blank plus 3 times the standard deviation of the

blank. The working range was determined to be the interval between 20% and 80% of the value of A1, as determined by the fit of the calibration curve.

Results and Discussion

Silicon photonic microring resonators arrays, each having 24 individually addressable sensors, were used for the real time monitoring of MCP-1. Microring sensors were functionalized to present either specific anti-MCP-1 capture antibodies or mouse IgG control antibodies (Figure 1a). Prior to the resonance measurements, substrates were loaded into a fluidic cartridge (Figure 1b) that was modified to allow incorporation of an array of neodymium magnets. Solutions containing different concentrations of MCP-1 were pre-incubated with a biotinylated tracer antibody and then flowed across the substrates through two channels that were defined by a laser cut Mylar gasket to assemble the sandwich assay. Freshly prepared magnetic bead solutions were then introduced to the flow cell. For magnetically actuated bead enhancement, the magnet was cycled in and out of the cartridge to actively drive the beads towards the sensor surface, while experiments performed in the absence of the magnetic field relied upon passive diffusion of beads to the surface (Figure 2). The flow channels were then returned to buffer and the net resonance shift due to the presence of magnetic beads was determined. The shift in resonance wavelength was monitored during each step of the assay, including sample/tracer binding, and during the bead-based signal enhancement step.

The primary goal of this study was to determine whether the implementation of a magnetic field to actively direct beads towards the microring sensor surface would lead to an improvement in assay metrics, specifically time-to-result, by avoiding the time needed for beads to passively diffuse to the sensor surface. Figure 3 shows the real-time resonance wavelength shifts accompanying the binding of magnetic beads, both through magnetic actuation (black trace) and passive diffusion (red trace). Using magnetic enhancement we observed a very rapid increase in the total resonance shift allowing the assay to be conducted in a total of 32 minutes. It is notable that at 32 minutes into the assay the response from the passive diffusion experiment was 9-fold lower than that of the magnetically actuated experiment. A much longer bead incubation (45 minutes) was needed to record a similar magnitude sensor response, which brought the total assay time to 73 minutes. Both of these assay formats are considerably faster than commercial available MCP-1 ELISA Kits (e.g., Pierce and R&D Systems), which require at least 3 hours.

In addition to assay rapidity, we also found that the magnetic enhancement strategy led to an improved limit of detection. Figure 4a shows calibration curves for both the magnetically-actuated and passive diffusion detection experiments across range of MCP-1 concentrations ($0 - 5 \text{ ng mL}^{-1}$). For the magnet-based enhancement (black points), the LOD was determined to be 57 pg mL^{-1} (working range: $385 - 5000 \text{ pg mL}^{-1}$). By comparison, after only 32 min total assay time the LOD for the passive diffusion experiment (red points) was higher— 215 pg mL^{-1} ($283 - 629 \text{ pg mL}^{-1}$). Figure 4b also shows a comparison between the 32 min magnet enhancement (black points) and the 73 min passive diffusion (blue points) experiments. While these two standard curves are more similar, the shortened assay featuring magnetic enhancement again had a lower LOD, with the 73 min assay LOD

being 124 pg mL^{-1} (working range: $401 - 1889 \text{ pg mL}^{-1}$). Notably, in both comparisons, the 32 minute magnet assay had a broader dynamic range than either passive diffusion detection experiment, which would be more useful for clinical diagnostic applications. The calibration curves were plotted with their corresponding error bars, which represent the standard deviation from $n=4$ replicate measurements. All the resonance wavelength shifts plotted in Figure 4 were determined after subtracting off the response of biological control sensors on the same chip, which were used to correct for non-specific adsorption. We did observe a small increase in the non-specific adsorption response in the magnetically-actuated detection experiment (see Supplementary Data); however, this is not surprising given that the magnet brings many particles into direct contact with the surface thereby providing a much greater opportunity for fouling to occur. The fact that the amount of non-specific response increase is proportionally much smaller than the increase in specific response is the basis for the improvement in assay limit of detection.

Comparison of our results with other previously reported methods to detect MCP-1 reveal advantages over competing methods, particularly when considering assay time and the relevant clinical concentration ranges. For example, Pasche reported a 90 min assay for MCP-1 (along with two other cytokines) with a 100 ng mL^{-1} limit of detection.²² Yu and co-workers recently developed an ultrasensitive electrochemical immunosensor for the detection of MCP-1 with a remarkable 0.03 pg mL^{-1} limit of detection and 60 min assay.²³ However, this sensor was limited to a maximum detection range of 360 pg mL^{-1} , which could possibly limit the clinical relevance of this device. In general, inflammatory cytokines need to be detected across a broad range from pg mL^{-1} up to ng mL^{-1} , and more specifically, the MCP-1 cut-off for utility as a prognostic ovarian cancer biomarker was reported to be 718 pg mL^{-1} .²⁴ By comparison, our magnetically enhanced immunosensor simultaneously offers a rapid assay (32 min), and a clinically relevant limit of detection (57 pg mL^{-1}) and dynamic range (up to 5 ng mL^{-1}).

Conclusions

This work demonstrates the integration of an array of magnets that can improve assay performance by actively pulling down magnetic beads onto a silicon photonic microring resonator platform. As a model system, we detected the inflammatory biomarker MCP-1 and found that the assay time-to-result can be dramatically reduced by $>56\%$ (73 min down to 32 min) compared to an assay that relied upon passive diffusion alone. We also observed a modest improvement in assay LOD, which was lowered from 124 to 57 pg mL^{-1} . While this proof-of-concept study focused on the detection of MCP-1, the methodology could be generally applied to any sandwich assay-type assay format for protein or even nucleic acid biomarkers of interest. Therefore, this technology could be broadly applied to the detection of other biomarkers of high clinical interest where sensitivity and assay time are important considerations, as is the case for many point-of-care diagnostic applications.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

a) Map of the distribution of spotted capture and control antibodies across the sensor surface, including thermal control microrings. The chip was divided into two fluidic flow channels. b) Top and side view of the fluidic cartridge components and magnet array.

Figure 2.

Overall strategy for the MP-enhanced detection of MCP-1. Comparison between the use of magnetic actuation to pull beads to the sensor surface and passive bead diffusion. The inset shows representative real time shifts in microring resonance accompanying bead binding in both a magnetically-actuated and passive diffusion detection experiment.

Figure 3.

Direct comparison of the effect of magnetic actuation on sensor response for the detection of MCP-1. In both traces a concentration of 5 ng mL^{-1} was detected and the responses are from three identical replicates with error bars indicating the standard deviation. The black trace shows the rapid increase in resonance shift measured when an array of magnets placed under the cartridge is used to actively pull beads to the sensor surface. The red trace (passive diffusion) shows a much longer amount is required.

Figure 4.

a) Calibration curves constructed based upon the magnetically-actuated (black squares) and passive diffusion (red points) enhancement strategies. B) Comparison of the 32 minute magnetically-actuated assay (black squares) with the 73 minute passive diffusion experiment (blue triangles). In all the cases, the number of replicates $n = 4$.