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Microfluidics integrated lithography-free nanophotonic biosensor for the detection of small molecules

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Currently, labeling techniques are mostly used to study the binding kinetics of small molecules and their detection (molecular weights < 500 Da) at low analyte concentrations. However, the fluorescent tags used in this method for conjugation may modify or prevent the functionality of the targeted molecules. Although label-free techniques provide more accurate quantitative and kinetic measurements by monitoring the binding of analytes in their usual forms, the monitoring of small molecule binding at lower concentrations is still impossible using conventional refractometric sensing devices. Here, we demonstrate a label-free and cost-effective technique for the detection of ultra-low molecular weight biomolecules such as biotin (244 Da) at very low concentrations. This extreme sensitivity is realized by utilizing the phase singularity feature of lithography-free nanophotonic cavities. The proposed four-layered metal-dielectric-dielectric-metal asymmetric Fabry-Perot cavity exhibits point-of-

darkness and phase singularity at the Brewster angle. Since the singular phase behavior depends on the topology of the cavity surface, it is very challenging to realize singular phase in reflection mode for a microfluidics integrated nanophotonic cavity. To overcome this issue, we integrate a specially designed poly methyl methacrylate microfluidic channel with the cavity. The experimental realization of real-time label-free biosensing of small molecules using lithography-free nanophotonic platforms can be used to develop cost-effective non-invasive liquid biopsies for point-of-care clinical evaluation, early cancer screening and real-time diagnosis of diseases.

In recent years, optical biosensors based on refractometric sensing devices^[1] have made a significant impact on the study of real-time biomolecular binding affinity and kinetics, since that can measure the changes in refractive index which occurs in the field of an electromagnetic wave supported by the optical structure of the sensor. In general, the performance characteristics of a biosensor is defined as its ability to detect a biomolecule that binds on the sensor surface. However, it is very challenging to acquire a detectable signal if the bound molecules are ultra-low molecular weight biomolecules (< 500 Da) at low analyte concentrations. Therefore, labeling techniques have been used to study the small molecules binding at low analyte concentrations^[2-4]. However, in the context of single molecule sensing and emerging point-of-care applications, labeling techniques are not preferred because the conjugated fluorescent tags may structurally and functionally interfere with the targeted molecules. In this regard, a number of label-free optical techniques have been developed for the study of real-time binding of analytes, even at single molecule level^[5-12]. Surface plasmon resonance (SPR)-based biosensors^[13-15] are the one of the most powerful label-free and refractometric biosensors, which can be used to detect the real-time binding of biomolecules with the help of bio-receptors functionalized on a thin metallic film or a metallic nanoparticle. The plasmonic resonance supported by many engineered nanostructures including metamaterials have also been proposed for improving the detection limit of plasmonic

biosensors^[16-21]. Although conventional SPR-biosensors and plasmonic biosensors-based on metamaterials can detect highly diluted analytes, the low sensitivity of these sensors fail to detect dilute analytes of low molecular weights or low surface coverage of very small bound molecules.

Over the past years, different optical systems with high sensitivity have been proposed for the detection of small molecules^[22-26]. In particular, plasmonic biosensing with an extreme sensitivity has been achieved using hyperbolic metamaterials (HMMs), which is capable of detecting very small molecules at lower concentrations^[22-24]. HMMs are artificial uniaxial materials that exhibit hyperbolic dispersion because the out of plane dielectric constant has an opposite sign to the in-plane dielectric^[27]. However, these systems rely on intense nanopatterning techniques, which is not cost-effective and restricts the scalability of the samples. Even though lithography-free planar systems have been demonstrated for small molecules sensing^[28-29], the *detection limit* of those systems are less than that of nanopatterned optical systems.

It was demonstrated that phase-sensitive interferometry techniques show higher sensitivity compared to sensors based on spectroscopic techniques^[30-32]. Our recent investigations have shown the confirmation on the existence of point-of-darkness and extreme phase singularity in a lithography-free nanophotonic cavity^[33-35]. We experimentally demonstrated that phase-sensitive biosensing was possible using a four-layered metal-dielectric-dielectric-metal (MDDM) thin film with one of the dielectric layers being an ultra-thin layer of germanium (Ge)^[33]. This asymmetric Fabry-Perot cavity exhibits *perfect light absorption* and *singular phase* behavior at the point-of-darkness, which are dependent on the existence of strong optical losses in Ge amplified by the cavity resonance. Since the point-of-darkness and phase singularity depend on the topology of the cavity surface, the realization of singular phase in reflection mode using a microfluidic channel integrated cavity system is a challenging task. Therefore, we did not demonstrate the real-time binding and sensing of

biomolecules in our previous report^[33] due to the absence of point-of-darkness and singular phase with a polydimethylsiloxane (PDMS) microfluidic channel integrated cavity system.

Here, we demonstrate the existence of *point-of-darkness* and singular phase using a specially designed poly methyl methacrylate microfluidic channel integrated nanophotonic cavity. By using this sensor platform and the well-known streptavidin-biotin affinity model, we show the real-time binding and sensing of small biomolecules such as biotin at very low concentrations. The detection limit achieved for this cavity system through phase-sensitive interrogation scheme is comparable with that of nanopatterned HMM-based biosensors, where the sensing mechanism was based on spectral wavelength shift. The importance of the detection of biotin is that it is a model system for small molecule compounds such as vitamins, cancer-specific proteins, hormones, and therapeutics.

A schematic representation and photograph of the fabricated microfluidic channel integrated lithography-free four layered nanophotonic cavity is shown in **Figure 1a** and **Figure 1b**, respectively. The asymmetric Fabry-Perot cavity consists of a silicon substrate, a top gold (Au) layer with thickness of 20 nm and bottom silver (Ag) layer with thickness of 80 nm forming the cavity mirrors. An ultrathin layer of Ge with thickness 10 nm was deposited on the bottom Ag film and a lossless relatively thick MMA layer was spin coated on Ge. Here, MMA layer acts as the cavity so that the thickness of MMA layer determines the number of cavity modes as well as the spectral positions of the modes. To obtain the cavity resonances in the visible wavelengths, the thickness of MMA layer was chosen to be 520 nm. Importantly, the Ge layer should be ultrathin in order to realize point-of-darkness and singular phase because a thick Ge layer alter the conditions for critical light coupling to the resonator, which minimizes the light trapped inside the cavity^[33-35]. We selected top metal layer as Au because the biomolecules can be easily functionalized with Au using a thiol-based surface chemistry^[36]. The thin films of Au, Ag and Ge layers were deposited by means of thermal

evaporation (Oerlikon Leopold vacuum system) of Au, Ag and Ge pellets at a deposition rate of 0.2 Å/s, 0.5 Å/s and 0.1 Å/s, respectively and a common base pressure of $<5 \times 10^{-6}$ mbar. The desired thickness of MMA layer was achieved by spin coating at 3000 rpm (8.5MMAEL 11, MICROCHEM). The thickness and optical constants of all thin films (see Figure S1 in the Supporting Information) were obtained using spectroscopic ellipsometry (J. A. Woollam Co., Inc, V-VASE).

Microfluidic channels were composed of a poly(methyl methacrylate) plastic top (encompassing micromachined inlets and outlets) and a double-sided 50- μ m adhesive film defining the outlines and thickness of the microchannels. PMMA tops were prepared by laser micromachining an inlet and outlet (diameter 0.61 mm, separation 14 mm) (Epilog Laser, Scottsdale, AZ). Optically clear double-sided adhesive tape (Thorlabs, Newton, NJ) was laser micromachined to encompass the 16 mm x 3 mm microchannels with 50 μ m height. The micromachined tape was attached to the PMMA component to include the inlet and outlet between the outline of the channels. The nanophotonic cavity was then assembled with the PMMA-film structure to form microfluidic channel integrated with the sensor. Inlet and outlet tubings (Tygon tubing, Cole-Parmer, Wertheim, Germany) were attached to the PMMA top and sealed using 5-minute epoxy (Thorlabs, Newton, NJ) for injection and removal of liquid biosamples.

A variable angle high resolution spectroscopic ellipsometer was used to measure the pair of ellipsometry parameters such as ψ (ellipsometric reflection) and Δ (phase shift). The recorded ψ and Δ spectrum of the cavity (without channel) as a function of excitation wavelength at 65° incident angle is shown in **Figure 2a**. The fabricated cavity supports two cavity modes in which second mode that has resonance at 720 nm shows point-of-darkness and singular phase. As shown in **Figure 2c**, we obtained the point-of-darkness and singular phase at 65° in angular scan by selecting the excitation wavelength of 720 nm. We further

validated the experimental results using a numerical model based on transfer matrix method (see Figure S2 in the Supporting Information). The ψ and Δ spectrum of microfluidic channel integrated cavity in wavelength and angular scan is shown in **Figure 2b** and **Figure 2d**, respectively. It should be noted that the point-of-darkness and singular phase are obtained at lower incident angle (57°), as a result the resonance of the second mode is red shifted to 770 nm wavelength. This is due to the change in the topology of the cavity surface. It is important to note that the obtained ψ_{min} value and phase change at the point-of-darkness are almost same as that of cavity alone. In particular, near singular phase is achieved for the microfluidics integrated cavity. Nevertheless, this is sufficient for biosensing applications because it is not directly possible to use the extreme singular phase for biosensing due to the absence of light and abrupt phase change^[30]. In the following sensing experiments, we consider angular scan with a step of 0.5° to demonstrate the extreme phase sensitivity of the microfluidics integrated nanophotonic cavity.

As a first step, a standard sensor calibration test with known refractive index analytes was conducted to determine the detection limit of the sensor device. For this case, different weight ratios (0.25-2.5% w/v) of aqueous solutions of glycerol were injected into the sensor microchannel (volume= 2.4 μ l) and recorded the corresponding ψ and Δ spectrum as a function of incident angle. In **Figure 3a**, we plot the ψ and Δ spectrum of distilled (DI) water and 2% glycerol in DI water. As can be seen, a considerable shift in Δ is obtained close to the point-of-darkness (ψ_{min}), which is due to the change in refractive index of analyte solution. However, the measured ψ and angle (θ) shifts were negligible. The measured marginal phase shift ($\delta\Delta = |\Delta_{water} - \Delta_{glycerol}|$) with change in concentration of glycerol is shown in **Figure 3b**. A linear variation in phase shift with increase in refractive index of analyte is obtained. We then performed transfer matrix method-based numerical simulations to demonstrate the phase shift for the bulk refractive index changes. As shown in **Figure 3b** (black line), the simulated

phase shifts exactly match with experimentally realized results. The best fit was obtained for these parameters: $\lambda = 770$ nm, $\theta = 55.005^\circ$ and thickness of Au, MMA, Ge and Ag were 20 nm, 510 nm, 10 nm and 75 nm, respectively. The measured bulk refractive index sensitivity with respect to DI water and lowest glycerol concentration (0.5 % w/v) is, $\delta\Delta/\delta n = 3333$ deg./RIU, which is comparable with the angular sensitivity of nanopatterned HMMs^[24]. The bulk refractive index sensitivity of the proposed device can be further improved by performing the sensing at the extreme singular phase ($\psi_{min} < 1$). We numerically confirmed that about 5 fold improvement in sensitivity is possible if the sensor works at the extreme singular phase (see Figure S4 in the Supporting Information).

To demonstrate the label-free detection of small biomolecules in highly dilute solutions, we used a well-known streptavidin-biotin affinity model. In order to capture biotin, streptavidin molecules were functionalized on the top Au surface using a thiol-based surface chemistry^[22-24] (see Supporting Information). The sensor has the ability to measure the refractive index change caused by the capture of biotin molecules at the sensor surface within the microfluidic channel because a strong decaying field along the superstrate is possible^[33]. The performance of the sensor was monitored as a phase shift in the Δ spectrum by injecting different concentrations (1 pM to 1 μ M) of biotin prepared in phosphate-buffered saline (pbs) into the sensor microchannel. In **Figure 4a** and **Figure 4b**, we show the recorded ψ and Δ spectrum of different concentrations of biotin. Initially, the spectra of pbs were recorded, and then different concentrations of biotin were recorded after a reaction time of 30 min. Note that pbs was introduced into the microchannel to remove the unbound and weakly attached biotin molecules before each injection of a new concentration of biotin sample. It is evident from **Figure 4b** that phase value increased with increase in biotin concentration. The response of the device during the detection of 1 nM biotin is shown in Figure S5 in the Supporting Information, where we plot the Δ spectrum as a function of incident angle over time. A

notable increase in phase value over time implies the specific binding of biotin molecules on the sensor surface.

The sensor performance with different concentrations of biotin is shown in **Figure 4c** and **Figure 4d**. We analyzed the sensitivity of the phase shift to the number of adsorbed molecules on the sensor surface. We consider the saturation values of the phase shift for different concentrations c of biotin. In particular, the phase shift depends on the number of bound molecules $N(c)$ in the sensing region. Note that we cannot directly measure the accurate value of $N(c)$, however, we can estimate an upper bound $N_{\max}(c)$ based on the sensor parameters, such that the actual $N(c) \leq N_{\max}(c)$ for all concentrations ^[23-24]. According to our model, the obtained $N_{\max}(c) = 29c \times 10^{15} \text{ M}^{-1}$ (see Supporting Information). In **Figure 4c**, we plot the sensitivity of the phase shift ($\delta\Delta = |\Delta_{pbs} - \Delta_{biotin}|$) to the number of adsorbed molecules for the corresponding concentration of biotin. The experimental data points were fitted using a phenomenological double-exponential fitting function, $N_{\max} = A_1(e^{\delta\Delta/\beta_1} - 1) + A_2(e^{\delta\Delta/\beta_2} - 1)$, where $A_1, A_2, \beta_1, \beta_2$, are fitting parameters and $\delta\Delta$ is the phase shift. The best fit was drawn as solid curve with fitting parameters were $A_1 = 3.1 \times 10^3$, $A_2 = 1.5$, $\beta_1 = 0.5^\circ$, and $\beta_2 = 0.28^\circ$. **Figure 4d** represents the same data and curve as in 4c, but plotted in terms of the sensitivity, $\delta\Delta/N_{\max}$ with $N_{\max}$. It is clear that the ratio, $\delta\Delta/N_{\max}$ decays roughly like $1/N_{\max}$ with increasing $N_{\max}$, which implies that sensitivity of the sensor decreases with increase in biotin concentration. This exponential fitting function is chosen such that the limiting behavior at small $\delta\Delta$ is linear, as we would expect at lowest biotin concentrations (picomolar). By taking into account the phase resolution of the VASE ellipsometer, the detection limit that we achieved is 1 pM. The detection limit of the device can be further improved by performing the phase-sensitive sensing at $\psi_{min} < 2$. In addition, detection limit of the sensor can be improved by using a Goos-Hänchen shift-based

interrogation scheme since it is experimentally confirmed that enhanced Goos-Hänchen shift is possible at the singular phase of the nanophotonic cavity^[35].

In summary, we experimentally showed that a microfluidic channel integrated lithography-free planar nanophotonic cavity offers extreme sensitive label-free biosensing for the detection of small biomolecules at very low analyte concentrations. We demonstrated that bulk refractive index sensitivity of 3333 deg. /RIU is possible using the phase-sensitive sensing mechanism, allowing the detection of lower molecular weight biomolecules at low analyte concentrations. We further demonstrated that the developed sensor device has the capability to detect small biomolecule binding such as biotin at a concentration as low as 1 pM, which is comparable with the detection limit of nanopatterned hyperbolic metamaterials^[23-24]. Since the developed sensor device is based on a lithography-free nanophotonic platform, the sensing area can be significantly enlarged and fabrication costs can be dramatically reduced, which allows the development of multi-channel sensors for cost-effective point-of-care applications. In particular, the proposed device is a potential platform for developing cost-effective non-invasive liquid biopsies for the detection of exosomes^[37] for early cancer screening and real-time diagnosis of diseases, since no current technique has been integrated with standard bio-analytical systems for simultaneous profiling and quantification of the exosomes.

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

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Figure 1. (a) A schematic of fabricated microfluidic channel integrated four layered nanophotonic cavity. The thickness of Au, MMA, Ge and Ag layers are 20 nm, 520 nm, 10 nm and 80 nm, respectively. (b) Photograph of a two-channel sensor device with microfluidic channel and sample tubing.

Figure 2. Experimentally obtained ellipsometry parameters (ψ and Δ) as a function of wavelength (a) without channel at $\theta = 65^\circ$ and (b) with channel at $\theta = 57^\circ$. Experimentally obtained ellipsometry parameters (ψ and Δ) as a function of incident angle (c) without channel at $\lambda = 720$ nm and (d) with channel at $\lambda = 770$ nm.

Figure 3. Sensor calibration by injecting different concentrations (0.25 %-2.5% w/v) of glycerol in DI water (a) Measured ψ and Δ spectrum of DI water and 2% glycerol in DI water. (b) Plot of marginal phase shift ($\delta\Delta = |\Delta_{water} - \Delta_{glycerol}|$) with increase in refractive index of glycerol in DI water. The angular resolution of the instrument is set to 0.5° . The size of the data points (red squares) in **b** represents the error bar.

Figure 4. (a) Measured ellipsometry parameters (ψ and Δ) of the sensor device for different concentrations (1 pM to 1 μ M) of biotin in PBS. (b) Enlarged plots of Δ spectrum close to ψ_{min} for different concentrations of biotin in PBS. The angular resolution of the instrument is set to 0.5°. (c) The maximum number of biotin molecules (N_{max}) adsorbed in the illuminated sensor area versus the corresponding phase shift ($\delta\Delta$) and (d) The plot of the sensitivity $\delta\Delta/N_{max}$ versus N_{max} . Each square (red) represents a different initial concentration of biotin, as indicated by the labels. The curve (black) is a best-fit function, described in the text. The size of the data points in **c** and **d** represents the error bar.