Self-Referenced Silicon Nitride Array Microring Biosensor for Toxin Detection Using Glycans at Visible Wavelength

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ABSTRACT

We report on application of on-chip referencing to improve the limit-of-detection (LOD) in compact silicon nitride (SiN) microring arrays. Microring resonators, fabricated by e-beam lithography and fluorine-based etching, are designed for visible wavelengths (656nm) and have a footprint of 20 x 20 µm. GM1 ganglioside is used as the specific ligand for recognition of Cholera Toxin Subunit B (CTB), with Ricinus Communis Agglutinin I (RCA I) as a negative control. Using micro-cantilever based printing less than 10 pL of glycan solution is consumed per microring. Real-time data on analyte binding is extracted from the shifts in resonance wavelengths of the microrings.

Keywords: Sensing, Microring resonator, Silicon nitride, Glycan, Cholera Toxin Subunit B, Ricinus Communis Agglutinin I

1. INTRODUCTION

On-chip photonic devices provide sensitive transduction mechanisms for biochemical sensing. Various devices such as 1D1 and 2D2 photonic crystals, travelling wave resonators,3–6 surface plasmon resonance based sensors,7,8 interferometers9 (including Mach-Zehnder,10 Young,11,12 Hartman,13 and backscattering14 interferometers), and resonant mirror structures15 have been employed for different sensing scenarios. On-chip travelling wave resonators (TWR) (also known as whispering gallery mode resonators) provide sensitive and relatively miniature devices,5,16–20 while maintaining simplicity of the structure. Practically, this simplicity translates to higher fabrication tolerance and yield. The possibility of attaining high quality factor TWR on chip,5,6,17,21,22 has encouraged demonstration of label-free single molecule detection using on-chip toroids5 and spheres,6 as an alternative to labeled methods such as fluorescent techniques.23 On the other hand, miniature size of these cavities enables realization of large microarrays in a compact chip,24 as well as high light-matter interaction due to their high confinement of the light and small mode volume.25 While ultra high quality factor (Q) resonators provide ultimate sensitivities down to a single molecule, they entail stringent conditions on the system design with regard to optical, electrical, thermal, and biochemical interferences. Thus depending on the application, higher Q can be compromised for other system merits such as compactness or reliability. For applications in blood biomarker screening or environmental sensing, abundant biochemical interference necessitates appropriate surface functionalization or analyte filtering to match the ultra high sensitivity of the resonator, which is still a challenge in current state of the art.

Silicon nitride as a CMOS compatible material has been of interest for realization of optical and electronic integrated systems. It is low-loss at visible wavelengths22 and has a small thermo-optic coefficient (TOC) compared to silicon. Furthermore, transparency at visible wavelengths enables utilization of visible light that

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can be detected by inexpensive silicon detectors. On the other hand, the small TOC reduces thermal drift of the resonators, making the device more tolerant to thermal variations across the chip.

Label-free sensing methods avoid the time and cost associated with sample preparation steps. Labeling the target can lead to complications in terms of steric hindrance or conformational strains on the proteins. Furthermore the number of tags per target molecule cannot be accurately controlled in many cases, which makes estimation of the concentration imprecise. Among different label-free detection schemes, glycans offer wide structural diversity and relevant evolutionary background, so as to make glycomics a candidate for the third class of informative macromolecules, together with nucleic acids (genetics) and proteins (proteomics). Glycans can be used for label-free monitoring of toxins and blood biomarkers, using the same binding mechanisms occurring on the cell surface. Ganglioside GM1 is one of the well-known glycans playing a central role in the attachment of simian virus 40 (SV40) and the toxins derived from V. cholerae and E. Coli. Cholera toxin has an AB5 structure, with B subunits responsible for attachment of the toxin to the cell through their specific binding to the GM1 ganglioside on the cell surface.

Here we have used an array of on-chip silicon nitride microrings for label-free toxin detection. Two type of reference microrings, covered by an oxide layer and exposed to sample, are employed to evaluate and compensate for thermal variations, laser drift, and non-specific binding. Resonances of the reference and sensing microrings are multiplexed in spectral domain while all of the microrings are coupled to a single waveguide. The option of covering the reference microrings with SU-8, instead of oxide, is investigated and the surface properties of these two type of cover layers are compared with regard to the contingencies of micro-cantilever based printing technique. In addition to the specific molecular receptors for capture of the target molecules, the sensor benefits from a poly(ethylene glycol) brush in order to minimize the non-specific binding. Real time binding of Cholera Toxin subunit B (CTB) to GM1 is demonstrated, with Ricinus Communis Agglutinin I (RCA I) as a negative control.

2. DESIGN, FABRICATION AND CHARACTERIZATION

2.1 Design and Simulation

Single-mode silicon nitride microrings are designed for sensing at 652-660 nm window. Resonance of the microring is sensitive to any environmental change. Hence by functionalizing the surface of the microring so as to only capture target molecules, the resonance shift will be proportional to the mass of the captured target molecules. Microdisk cavities, as an alternative to microrings, have higher quality factors since the mode suffers from roughness of only one sidewall rather than two. However the plurality of the microdisk modes crowds the spectrum, practically making tracking the modes impossible in spectrally multiplexed systems. This problem may be worked around by designing highly selective coupling methods, such as pulley coupling, at the expense of lower tolerance to fabrication imperfections.

Microrings are critically coupled to a bus waveguide at their resonance wavelength. The critical coupling results in maximum extinction and higher signal-to-noise ratio (SNR) in detection of the resonance shift. We choose not to undercut the devices to have higher mechanical stability and fabrication yield. The device consists of five such microrings coupled to a single waveguide, as illustrated in Figure 1. We use COMSOL for finite-element simulation and the design of the microrings.

Microrings with a radius of 8 \( \mu \)m and a width (w) of 500 nm are fabricated in a 240-nm-thick silicon nitride film (Figure 1). The substrate is a 4 \( \mu \)m oxide layer on a silicon wafer. The choice of 8 \( \mu \)m radius ensures enough spectral room for multiplexing the microrings while maintaining a high quality factor. The width is designed to render the microrings single-mode. In the single-mode width range, the lower widths push the mode profile out of the waveguide, making the microring more sensitive to environmental changes. However, at the same time, the lower widths reduce the quality factor and its homogeneity from one microring to another. As the proper gap for critical coupling depends on the quality factor, it is important to have consistent quality factor to make sure all the microrings are critically coupled to the waveguide.

The structure has a TE and a TM mode around 656 nm wavelength. Bulk sensitivity of the microring is 29 nm/RIU for TE and 48 nm/RIU for TM mode, according to COMSOL simulations. Surface sensitivity is 0.58 nm/nm.RIU for TE and 1.1 nm/nm.RIU for TM mode (i.e., 1.1 nm shift per 1nm of the deposited film with
refractive index of 2.33, which is 1 unit more than the refractive index of water). Due to its higher sensitivity we have picked the TM mode for sensing, which has an FSR of 4.0 nm. The penetration depth of the TM mode into the water cladding is about 90 nm. The microrings are placed 600 pm apart in spectral domain by changing their radii. Two of the microrings are covered with an oxide film. These microrings will serve as references to cancel thermal drift and laser instability. Two of the microrings will be coated with GM1 glycan. The last microring, also exposed to the sample, will monitor the level of non-specific binding.

2.2 Nanofabrication Methods

The devices have been fabricated and processed at Georgia Institute of Technology’s cleanroom facility. Starting from silicon wafer, growth of 4 µm thermal oxide and deposition of 240 nm LPCVD silicon nitride was performed by LioniX™. Cleaved pieces of the wafer are cleaned by acetone, methanol, and isopropanol rinse. The chips are dehydrated for five minutes at 100°C on hotplate and spin coated after cooling. ZEP520A is spun at 1500 RPM for 1 minute, and then baked at 180°C for two minutes. Coated chips can be kept in a dark place for a few weeks. Right before electron beam lithography (EBL), ESPACER was spin coated at 4000 RPM for 30 seconds on the pieces, to reduce charge-up effect. Device patterns are written using JEOL JBX-9300FS EBL System into ZEP, with a current of 2 nA and dosage of 200 µC/cm². Before developing, the chips are rinsed with running deionized (DI) water for 1 minute to wash the ESPACER layer. Next they are developed in amyl acetate for 2 minutes with gentle agitation, and then soaked in isopropanol for 30 seconds. Dried chips are etched in Oxford endpoint RIE etcher using CF₄ plasma (20 sccm CF₄ flow, 200 W power, 10 mTorr pressure). Residual ZEP is removed after the etch by soaking the chips in Microposit remover 1165 at 80°C for 20 minutes, followed by acetone rinse.

The devices at this stage are optically functional and they can be characterized to ensure acceptable quality of fabrication before proceeding to the next step. Although optical or scanning electron microscopy help to visually inspect the fabricated devices, checking the critical coupling is only possible through coupling laser light into waveguides and measuring the spectrum. Next, Shipley 1813 is patterned on the chip to cover the sensing microrings. Shipley 1813 is spin coated at 500 RPM for 10 seconds and then 4000 RPM for 60 seconds followed by 1 minute bake at 115°C on hotplate. Photolithography is done using 365 nm UV light with a dosage of 150 mJ/cm². Subsequently the chips are developed in Microposit MF-319 for 50 seconds with gentle agitation, and then rinsed in DI water. One minute descum in oxygen plasma removes the residual resist or contaminations.

Figure 1. Microring sensor layout. (a) Structure of the sensing element. The inset shows radial electric and magnetic fields of TM mode at the microring cross section. (b) Layout of the exposed and covered microrings in the array. The covered microrings monitor thermal drift of the resonances and laser instability, while the uncovered microrings monitor binding events.
and enhances adhesion of evaporated oxide to the nitride film. CHA e-beam evaporator is used to deposit 1 µm of oxide on the chips. E-beam evaporation provides lower side-wall coverage compared to PECVD and is more suitable for oxide lift-off. After 10 minutes of submerging the chips in acetone, gently brushing the surface with an acetone soaked swab helps to detach residues of the sacrificial layer. As a result of this process, the chips are covered with an oxide film having windows opened on top of the corresponding microrings (Figure 2(c)).

Use of an ESPACER layer facilitates the dissipation of the electrons during EBL and prevents charge-up. Insulating properties of silicon nitride does not let the electrons spread throughout the surface easily. The accumulated charge can effectively interfere with the EBL writing process, deflecting the electron beam and displacing some segments of the pattern. Figure 2 shows examples of devices fabricated with and without application of ESPACER. In Figure 2(b) due to severe charge-up, electrons have been drastically deflected in some segments of the patterns. These segments appear as horizontal lines in the SEM of Figure 2(b), which have not received enough dosage of energy and so have not been removed in the developer.

We also investigated the option of cladding the reference resonators using SU-8 2005 (MicroChem) photoresist, as an alternative to oxide clad. The advantage of using SU-8 is its relatively simpler process, which alleviates the need to evaporate oxide as well as conventional lift-off issues. SU-8 is spin coated at 500 RPM for 10 seconds, followed by a 30 second spin at 2000 RPM. Solvent is evaporated by a soft bake at 95°C for 2 minutes. Pattern of opening windows are exposed by a dosage of 150 mJ/cm² with 365 nm UV light. Post exposure bake is carried out at 95°C for 3 minutes, and samples developed in SU-8 developer for 90 seconds. The sample is rinsed with DI and hard baked at 250°C for 30 minutes, to make SU-8 resistant to Piranha cleaning used in the subsequent surface chemistry step. Hard bake also saturates epoxide functional groups, otherwise they are capable of capturing proteins or any amine containing molecules through epoxide's reaction with -NH group of amine.

2.3 Functionalization and Receptor Immobilization

2.3.1 Materials

BS(PEG)9 crosslinker was purchased from Pierce (Rockford, IL, USA) and anhydrous DMSO from Acros Organics (Pittsburg, PA, USA). All other solvents and chemicals were obtained from Sigma-Aldrich (St. Louis, MO, US).

2.3.2 Glycan Immobilization

The chips are cleaned in Piranha (5:1 H₂SO₄ and H₂O₂) for 10 minutes and then rinsed with DI water. They are dehydrated for 10 minutes on a hot plate at 100 °C. After cooling, they are put in 5% (3-aminopropyl)-triethoxysilane (APTES) in anhydrous ethanol for one hour. The chips are thoroughly rinsed with anhydrous ethanol and dried with nitrogen stream. The APTES film is cured at 100°C for 20 minutes, to stabilize the
siloxane bonds and reduce their hydrolysis rate in aqueous solutions.\textsuperscript{38, 39} Silanized chips are then placed in a Petri dish and their surfaces are covered with drops of 1% w/v BS(PEG)\textsubscript{9} in anhydrous DMSO. High concentration of this homobifunctional crosslinker is necessary to ensure high competition between crosslinker molecules and having majority of them attached only at one end to the surface. The Petri dish, sealed with Parafilm, is left undisturbed at room temperature for 5 hours. Afterwards, the NHS-functionalized surfaces are washed in anhydrous ethanol thoroughly and dried by nitrogen stream. The poly(ethylene glycol) spacer arm in BS(PEG)\textsubscript{9} crosslinker decreases non-specific binding of any molecules attaching to the surface with binding mechanisms other than the specific glycan-lectin binding. The spacer arm is 3.6 nm, which is much shorter than the 90 nm penetration depth of the TM mode into the water clad. NHS esters are stable at -20°C for several months. However in our process we always immobilized glycans right after NHS functionalization. The 200 mM solution of the amino-derivatized glycan\textsuperscript{40} (GM1) is locally spotted on the corresponding microring using BioForce Nano eNabler printer. The tool is able to dispense about 10 pL of the solution using its 30 µm tip, just enough to cover one microring. The glycan solution is mixed 1:1 with phosphate buffer (300 mM, pH 8.5), containing 5% glycerol for printing. The glycerol is added to slow down the evaporation of the drop. After the print, the chips are incubated in high-moisture chamber at 50°C for 1 hour, and then dried over night. The dried surface is thoroughly washed in 0.05% tween20 in phosphate buffered saline (PBS), followed by DI water wash. Remaining NHS esters, neither occupied by glycan molecules nor hydrolyzed, are blocked by 50mM ethanolamine in 0.1 M Tris buffer, pH 9.0, for 1 hour. The blocking step prevents nonspecific capture of toxins on the surfaces other than those of microrings.

2.4 Optical Characterization
Focused laser light is side-coupled to the waveguides at the edge of chip. Laser source is a New Focus TLB 6305 tunable laser module sweeping 652-660 nm window. Laser source is conditioned by a half-wave plate and a polarizing beam splitter to rotate the polarization of the laser output onto TM direction and suppress the TE component. The device is aligned to have the edge of the input waveguide in the focused laser beam. On the other end, another lens is used to project the output light onto a silicon detector. A polydimethylsiloxane (PDMS) reservoir is placed on top of the microrings to contain the sample solution. A LabView module controls the laser sweep and records the detector reading. Collected spectral data is analyzed for detecting the resonances and fitting a Lorentzian to the detected resonances.

3. RESULTS AND DISCUSSION
We investigated oxide as well as SU-8 clad layer to cover the reference microrings (Figure 1(b)). Despite its benefits, we found SU-8 to be considerably more wettable than silicon nitride and silicon dioxide after NHS chemistry. This fact together with the capillary action of the waveguide trenches leads to displacement of the glycan drops spotted locally on the microrings (Figure 3(a)). As the glycan molecules need about one hour incubation time to form a dense layer on the substrate, displacement of the drops results in poor surface coverage by the glycans. It is possible to make SU-8 nonwettable, by depositing a fluorosilane monolayer on SU-8 right after exposure (and before development). Figure 3(b) illustrates contact angle measurement for fluorosilane treated SU-8 and regular silicon surface. However, this monolayer did not prove to be resistant enough to Piranha or oxygen plasma, needed for our surface chemistry. As a result we utilized the oxide clad protocol in our processes.

The transmission spectrum of the fabricated device, while covered with water, is shown in Figure 4(a). There are two resonances for each microring, separated by an FSR of 4.0 nm. Binding of the toxin molecules to the functionalized microrings can be monitored in real time using continuous scanning of the device. Initially 10 consecutive measurements were performed under water clad to evaluate the 3σ noise for the resonance detection. With proper referencing the noise was around 1 pm. Our external cavity laser mechanically rotates an internal grating to tune the wavelength. It is worth mentioning that consistency of the wavelength reading of this laser is dependent on its return slew rate (the speed with which the grating returns to its initial setting). Under identical conditions, having the laser return at 9 nm/sec results in 3σ ≈ 70 pm, while for slew rates below 2 nm/sec these variations in apparent resonance wavelength are about 6 pm. Using the proper referencing, the variations (with either slew rate) reduce to 1 pm. With this resolution in the resonance tracking, the limit of detection is 400...
Figure 3. Consequences of SU-8 hydrophilic surface (a) Fabricated microring array with SU-8 cover. As opposed to flat areas, the drops on the microrings move toward SU-8 area. The arrows show the displacement route of the spotted drop on the microring. (b) Contact angle of water on SU-8 surface with fluorosilane monolayer, compared to regular silicon surface.

Figure 4. (a) Normalized transmission spectrum of the spectrally multiplexed microrings. (b) Sensor response to introduction of CTB and return toward baseline for negative control (RCA I).
ag for this system, which amounts to 8 pg/mm² (or equivalently 8 resonance units, RU). Figure 4(b) shows the referenced resonance shift of GM1 coated microring over time. The CTB was diluted with DI water down to a concentration of 1 μM and a drop of this sample (approximately 10 μL) was dispensed into the PDMS reservoir with micropipette. The reservoir was capped with a cover slip to hinder evaporation. After 45 minutes the chip is washed off-stage using 0.05% tween20 in PBS and then DI water. Dried chip was set up on the stage and 10 μL of 10 μM RCA I solution was introduced as a negative control, to which GM1 is not expected to demonstrate appreciable binding.

The microring shifts about 50 pm during the first 45 minutes. According to COMSOL simulations, surface sensitivity of the microring is 1.1 nm/nm.RIU. In other words, for an organic layer with a refractive index of 1.45 (which is typical for proteins), every 1 nm of the film should result in 130 pm shift. On the other hand, the molecular weight of CTB, 11.6 kDa, makes it comparable to the proteins with about 100 residues and size of 3 nm. In this condition, the 50 pm shift for 1 μM CTB solution amounts to surface coverage of about 25%, comparable to a closely packed CTB monolayer.

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