Real-time analysis of molecular conformation using silicon electrophotonic biosensors

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ABSTRACT: Silicon microring resonators are widely used as optical biosensors because of their high sensitivity and promise of low-cost mass-manufacturing. Typically, they only measure the adsorbed molecular mass via the refractive index change they detect. Here, we propose and demonstrate a silicon microring biosensor that can measure molecular thickness and density as well as electrochemical activity simultaneously, thereby enabling quantification of the conformation of surface-immobilized biological and molecular layers in real time. Insight into the molecular conformation is obtained by recording the resonance shift from two geometrically distinct ring-resonators connected to a single access waveguide. The resonant cavities both support a single TE polarized optical mode but have different widths (480 and 580 nm); the extent of their evanescent fields is thus very different providing different depth-resolution of the interaction with a molecular layer on the sensor surface. By combining the optical shift from these two measurements, we demonstrate unambiguous quantification of the thickness and the refractive index of a molecular layer assembled on the waveguide. The precision of the technique is 0.05 nm and 0.005 RIU in the molecular layer thickness and refractive index, respectively. We demonstrate the cascaded electrophotonic ring resonator system using two exemplar systems, namely a) physisorption of a bovine serum albumin monolayer and b) an electroactive DNA oligonucleotide hairpin, where we uniquely show the ability to monitor electrochemical activity and conformational change with the same device. This novel sensor geometry provides a new approach for monitoring the conformation and conformational changes in an inexpensive and miniaturized platform that is amenable to multiplexed, high-throughput measurements.

Keywords: photonic biosensing, cascaded ring resonators, electrophotonic biosensor, thickness and refractive index extraction

 The three-dimensional fold or conformation of a protein determines its function. Many important degenerative conditions, including Alzheimer’s disease, result from protein mis-folding. For disease diagnosis it is therefore important to not only measure the presence of a specific protein, but also its conformation. Furthermore, conformation is not always static. Many biological and molecular processes, for example the entry of a pathogen such as HIV into a target cell (1), critically depend on specific conformational changes. A number of biophysical techniques have been developed to study protein conformation and conformational change including X-ray crystallography (2), neutron reflectometry (3),(4), and nuclear magnetic resonance (NMR)(5). While X-ray crystallography offers very high spatial resolution, the need for a crystallized sample limits the ability to probe conformational changes as they occur in real-time. Both neutron reflectometry and NMR are capable of studying conformational dynamics, but they are not suitable for high throughput screening and require bulky and expensive equipment.

Label-free photonic biosensors have emerged as a promising, low-cost diagnostic platform for the specific and sensitive detection of protein biomarkers. The sensitivity of these techniques arises from the overlap of the evanescent optical field with a biological layer immobilized on the sensor surface. Changes in the density, D, and/or the thickness, *t*, of the layer are reflected in a change in the averaged refractive index of the layer, *n*, which is translated directly into the effective refractive index, *neff*, of the optical mode. In conventional, single mode evanescent wave sensors, it is not possible to disentangle changes in *n* and *t* from the single measurement of *neff* (a given change in *neff* could be either due to a thick layer of low average refractive index, or a thin layer with high refractive index). Recently, dual-polarization interferometry (DPI)(6) and dual-polarization silicon microring analysis(7) have been shown to enable deconvolution of *n* and *t* enabling quantification of conformational changes in surface-bound proteins. Here, two uncorrelated measurements of the molecular layer are performed (using the orthogonally polarized transverse electric (TE) and transverse magnetic (TM) fundamental modes). These modes have a different interaction with the surface-bound molecules due to differences in the lateral extent of their evanescent tails. Each mode thus experiences a different variation in *neff*. The change in *neff* can be modelled for each optical mode through a system of equations which can then be individually resolved to unambiguously determine both *n* and *t* (6),(7) Nevertheless, as TE and TM modes have different orientations, they sample different molecular directions, which adds uncertainty to the calculated layer properties especially when trying to determine conformation. Furthermore, these techniques require the sensor to be excited with both TE and TM polarizations, adding complexity to the design and operation of the sensor. The TE and TM modes are also fixed in a given system, which limits the available degrees of freedom for designing and maximising the light-matter interaction.

In this letter, we present an innovative label-free biosensor that is capable of quantifying conformation in biological and molecular layers with sub-nanometre resolution and in real time, using only a single polarization. As in dual-polarization silicon microring analysis, the technology is based on silicon photonic ring resonator biosensors (8), but we now use a cascade of two separate rings operating in the same polarization (see Figure 1). The rings are made of waveguides of different widths to control the evanescent tails of the respective resonant modes. The resonance wavelengths of each ring will therefore respond differently to a change in refractive index at the sensor surface which provides the required two-equation system to resolve both *n* and *t*. Furthermore, by selectively doping the surface of the silicon substrate it is also possible to perform electrochemical characterization of surface immobilized redox-active systems alongside photonic measurements (9). Here we exploit this capability to fully characterize the conformation of an electroactive DNA hairpin.

**Figure** 1**.** The cascaded photonic ring resonator biosensor. (a) Two ring resonators with different cavity widths are cascaded along the same access waveguide. (b) Simulation of the electric field distribution of the optical mode for each of the ring resonators at the cross section of the waveguides (air cladding). Two different waveguide widths (here 480 and 580 nm) are employed to control the overlap of the evanescent wave of each sensor with a molecular layer immobilized on the sensor surface. The discontinuity of the electric field distribution at the boundary of each waveguide originates from the need to satisfy the continuity of the tangential component of the electric displacement field at the interface. The origin of the graph in X-direction refers to the centre of each waveguide.

CASCADED CONFIGURATION

DESIGN AND FABRICATION. Ideally, the individual cavities in the cascaded ring resonator sensor should be designed to maximize the differences in evanescent fields in order to increase the orthogonality of the two-equation system. This can be achieved using resonators with a large difference in width. However, there are two important limitations to consider. Firstly, guidance of second order modes should be avoided as they decrease the Q-factor of the cavity, and thus limit the detection sensitivity. This condition is satisfied using a cavity waveguide with a width of less than 586 nm for a cavity length of around 200 µm (10). Secondly, the propagation losses increase for ring widths smaller than 360 nm, again leading to a reduction in the Q-factor of the cavity (8). We thus designed the cascaded ring resonator sensor with waveguide widths of 480 and 580 nm (Figure 1) and with cavity lengths of approximately 200 µm and 210 µm, respectively. Different cavity lengths were employed to avoid overlap between coinciding resonances and to ease identification of the respective resonance combs (Figure 2 (a)). The penetration depth (distance over which the energy density of the mode decreases by a factor 1/e into a water cladding layer) was calculated to be approximately 80 nm and 49 nm for the 480 nm and 580 nm resonators (with an air cladding), respectively (Figure 1 (b)) (see supporting information (SI)).

**Figure** 2**.** Performance of the cascaded ring resonator configuration. (a) Transmission spectrum of the cascaded ring configuration (air cladding). Resonance peaks can be identified via their free spectral range (FSR), which are 2.42 nm and 2.69 nm, for the 480 and 580 nm wide sensors, respectively. (b) Resonance wavelength shift of the cascaded ring configuration against changes in the bulk refractive index. Measurements were performed using a range of NaCl solutions of different molar concentration. Sensitivities of 65 nm/RIU and 43 nm/RIU are obtained, for the 480 and 580 nm wide sensors, respectively.

The device was fabricated in silicon-on-insulator (SOI) wafer (220 nm silicon thickness on a 2 µm layer of buried oxide) (SOITEC, France) with a rib waveguide profile (30 nm slab thickness). Surface doped substrates (n-type, average doping density of 7.5 x 1016 cm-3) were used for the combined photonic and electrochemical measurement, as discussed below and in (9). The Q-factor, sensitivity, minimum resolvable optical shift and limit of detection (LOD) of each ring resonator is given in table 1, which are comparable to similar ring resonators reported in the literature (7),(8),(9)).

|  |  |  |
| --- | --- | --- |
| Waveguide width | 480 nm | 580 nm |
| Penetration depth(air cladding) | 80 nm | 49 nm |
| Q-factor  | 50,000 | 40,000 |
| Cavity length | ≈ 200 µm | ≈ 210 µm |
| FSR | 2.42 nm | 2.69 nm |
| Bulk sensitivity  | 65 nm/RIU | 43 nm/RIU |
| Δλmin | 0.75 pm | 1.05 pm |
| LOD | 1.15x10-5 RIU | 2.45x10-5 RIU |

Table 1. Performance details of both electrophotonic microring resonators.

The Q-factor of both rings is found considering an air cladding layer, while the bulk sensitivity was measured by exposing the sensors to a range of NaCl aqueous solutions of different refractive index (Figure 2(b)).

THICKNESS AND REFRACTIVE INDEX EXTRACTION PROTOCOL. A two-equation system linking the wavelength shift for each sensor (Δλ480nm and Δλ580nm) is needed to extract the thickness of a molecular layer immobilized on the surface of each sensor. We followed the method described in (7) to model the interaction of the optical field with the overlayer, in which Δλ480nm and Δλ580nm are described by two functions *f(t,n)* and *g(t,n)*, respectively. First, a set of electromagnetic simulations is performed using COMSOL Multiphysics to calculate Δλ480nm and Δλ580nm as a function of *t* and *n* for a background of water (n=1.32) These simulations are then fitted to an analytical model to determine the wavelength shift as a function of *t* and *n* (see SI) (7). After fitting and retrieving all the parameters, a coefficient of determination R2 of 0.9992 is obtained for both waveguides, confirming the validity of the model (Figure 3(a)). Finally, the functions *f(t,n)* and *g(t,n)* are fitted to experimental measurements of Δλ480nm and Δλ580nm to obtain two continuous functions of the thickness and refractive index of the molecular layer (Figure 3(b)). The point at which these two functions intersect corresponds to the unique solution of the thickness and refractive index of the molecular layer (Figure 3(c)). We note that the accuracy of this model is critically dependent on the exact geometry of each waveguide. We therefore characterized the dimensions of each waveguide using AFM (BioScope Resolve 2, Bruker), and found values of 482 x 202 nm and 578 x 205 nm for the width and height of each ring resonator, respectively. Additionally, it is possible to perform an in-line calibration to calculate the geometry of the ring cavities with an accuracy of ± 2 nm in width and ± 1 nm in height (see SI). This calibration can be performed prior to an experiment to ensure accurate quantification of the molecular layer. The noise in wavelength measurement, defined as three times the standard deviation, was found experimentally to be 0.43 nm for the 480 nm waveguide and 0.38 nm for the 580 nm waveguide. Assuming that the noise follows a Gaussian distribution with a standard deviation of 0.43 nm (maximum error from both resonators), it is then used to determine the error for a simulated wavelength layer of *t* = 2 nm and *n* = 1.45 RIU. The error in *t* and *n* is therefore found to be 0.05 nm and 0.005 RIU respectively, calculated considering experimental wavelength errors (see SI). The accuracy of the thickness measurement achieved with our cascaded resonator is therefore comparable to that of a dual polarization microring (where the thickness resolution is typically 0.02 nm) (7) and an order of magnitude better than that achieved with DPI (resolution of 0.1 nm)(6),(11). The temporal limit of detection of this technique is given by the throughput of the employed optical instrumentation. In our system, a broadband ASE (Amplified Spontaneous Emission) source (1520-1620 nm) and an Optical Spectrum Analyser (OSA) are employed, yielding a temporal resolution of approximately 60 seconds.

**Figure** 3. Extraction of thickness and refractive index. (a) Simulations of the wavelength shifts for both waveguide geometries as a function of the thickness of a molecular layer (assuming a refractive index of 1.45 RIU for the molecular layer and 1.32 RIU for the surrounding aqueous solvent). A coefficient of determination R2 of 0.9992 was obtained for each waveguide. (b) Solving protocol by plotting *f(t,n)* = Δλ480nm and *g(t,n)* = Δλ580nm. The intersection point of both curves corresponds to the solution of the two-equation system, which in the example shown corresponds to a layer thickness of 3.5 nm with a refractive index of 1.485 RIU. (c) Summary of the process. The resonance dips from both sensors are first measured in real time. The corresponding wavelength shifts, Δλ480nm and Δλ580nm, are then fitted to the model, which is solved obtaining both the thickness (*t*) and the refractive index (*n*) of the molecular layer.

RESULTS AND DISCUSSION

PHYSISORPTION OF A BOVINE SERUM ALBUMIN MONOLAYER. As an initial test, we verified our method by monitoring the adsorption of bovine serum albumin (BSA). BSA is a protein used widely as a blocking agent in immunoassays to prevent non-specific adsorption (12), and has been studied previously as an exemplar for DPI (11) and dual-polarization silicon microring analysis(7). BSA (Sigma-Aldrich, UK) was dissolved in a 2 mM phosphate buffer solution, pH 3 to a concentration of 0.1 mg/mL. The pH of the buffer solution is set below the isoelectric point of BSA (pI = 4.7) to ensure that the protein will assemble in a thin, dense layer due to its electrostatic attraction with the substrate, coupled with electrostatic repulsion between proteins (7). Phosphate buffer pH 3 was initially injected over the sensor surface at a rate of 30 µL/min, until a stable resonance wavelength was obtained for each sensor (Figure 4 (a)). Subsequently, the BSA solution was injected over the sensor at 30 µL/min for 60 min and then left incubating for 30 min. The thickness and refractive index of the BSA layer is shown in Figure 4 (b)-(c), respectively. At equilibrium, we obtained a layer thickness of 1.26 nm ± 0.05 nm and a refractive index of 1.432 ± 0.005 RIU. These values agree well with those estimated for a BSA layer assembled on a dual polarization ring resonator (thickness of 1.4 nm and refractive index of 1.433 RIU) (7).

**Figure** 4. Real-time adsorption of BSA on a silicon surface monitored using a cascading ring resonator sensor. (a) The resonance wavelength of both 480 and 580 nm width ring sensors was monitored continuously while BSA molecules are adsorbed on the sensor surface at pH 3. (b)–(c) show the estimations of both thickness and refractive index of the layer, respectively.

MONITORING THE CONFORMATIONAL CHANGES OF AN ELECTROACTIVE DNA OLIGONUCLEOTIDE HAIRPIN. By combining the cascaded ring structure with our recently developed silicon electrophotonic sensor (9), we introduce the truly unique capability of monitoring electrochemical activity together with quantification of molecular conformation. This capability allows us to directly interrogate the dependence between molecular conformation (optical interrogation) and redox activity (electrochemical interrogation). We note, while combined electrochemical and optical measurements have been demonstrated using electrochemical-surface plasmon resonance (EC-SPR) (13), EC-SPR employs a single mode and it thus remains challenging to unambiguously quantify conformation (14). Here we employ redox labelled DNA hairpins (15),(16), as a model system to demonstrate our multi-modal measurement capability. These DNA hairpins change conformation upon binding to a complementary DNA strand, which leads to a shift in the electrochemical activity of the redox label. Although this change in redox activity is typically used to indicate a change in the structure of the immobilized DNA layer, electrochemical measurements alone cannot unambiguously confirm a change in structure or provide a quantitative measure of molecular conformation. Our technology precisely resolves this ambiguity and lack of quantitative analysis, *in situ*, by combining optical measurements of molecular conformation with complementary electrochemical characterization of redox activity.

Our model system consists of an electroactive DNA hairpin (iba GmbH) modified at the 3'- end with methylene blue (MB) as the redox probe. This oligonucleotide, named MB-DNA, undergoes conformational change upon exposure to a complementary DNA strand (named Catalyst) which binds to a short toe-hold thereby displacing the double-stranded neck-region (shown schematically in Figure 5). The 5'- end of MB-DNA strand is thiol modified, which can be bound covalently to a thiol (-SH) reactive surface. Accordingly, the silicon sensor surface was modified to create a thiol reactive Cu1+ surface (17) (see SI). The sequences of MB-DNA and Catalyst are given in the supporting information and were obtained from (18).

**Figure** 5. Illustration of a toehold-mediated conformational change of the MB-DNA. MB-DNA closed) The MB-DNA strand is immobilized on the sensors. MB-DNA open) Toehold-mediated displacement triggered by the ssDNA Catalyst strand results in a conformational change of the MB-DNA strand. Due to the high flexibility of the resulting single stranded region attached to the sensor surface, the MB-DNA collapses on the surface.

Following formation of the Cu1+ modified silicon surface (see SI), a solution of MB-DNA (in PB (pH 7)) at a concentration of 1 µM was injected over the sensor surface at 20 µL/min (Figure 5, MB-DNA closed), until a stable wavelength shift was observed after 50 mins indicating saturation. For simplicity, the absolute wavelength shift at the end of each step is shown for this experiment in table 2.

|  |  |  |  |
| --- | --- | --- | --- |
| Step | Absolutewavelength shift [nm] | Thickness*t* [nm] | Refractive indexn[RIU] |
| 480 nm sensor | 580 nm sensor |
| MB-DNA closed | 0.2784 | 0.2028 | 1.97 ± 0.05 | 1.427 ± 0.005 |
| MB-DNA open | 0.455 | 0.3158  | 2.21 ± 0.05 | 1.443 ± 0.005 |

Table 2. Optical monitoring response analysis of a toehold-mediated conformational change of the MB-DNA.

After resolving the two-system equation for the experimental data of table 2 (accounting for the presence of the MPTS-Cu layer, see SI), we find that the thickness and refractive index of the MB-DNA monolayer is $t$ = 1.97 ± 0.05 nm and $n$ = 1.427 ± 0.005 RIU, respectively. Assuming a uniform surface density, consistent orientation of the MB-DNA molecule and polymorphic orientation of the single stranded loop region (20), the angle of the molecules with respect to the silicon surface is calculated to be 21.2°. Such a high tilt angle suggests a low density DNA monolayer. This is confirmed by electrochemical measurements of the MB redox active label (Figure 6(a)-(b)). From the area under the MB reduction peak (at -0.5 V) we calculate the density of MB-DNA at the surface to be equal to 1 x1011 molecules/cm2. We note, quantification of molecular density using conventional (i.e. undoped) ring resonators, require assumptions to be made regarding the RI and structure of the surface immobilized molecules. Here, the density is obtained directly from the electrochemical information, highlighting the capabilities and benefits of our dual-mode electro-photonic technology.

Next, the MB-DNA functionalized surface was exposed to 1 µM of the invading strand, Catalyst, to catalyse toehold-mediated displacement and opening of the immobilized hairpin (Figure 5, MB-DNA open). Again, the invading strand was allowed to flow over the sensor surface until a stable wavelength shift was achieved. Cyclic voltammograms now show two reduction peaks following toehold-mediated displacement; a large peak at approximately -0.6 V and a shoulder at -0.48 V (Figure 6(c)). From the area under the two peaks, we calculate that the surface coverage is unchanged (9 x 1010 molecules/cm2), indicating no MB-DNA was released from the surface. The presence of two reduction peaks is indicative of two different species immobilized on the surface; MB-DNA in the closed conformation which is electrochemically reduced at -0.48 V, and MB-DNA in the open conformation which is indicated by the reduction peak at -0.6 V. After fitting two independent Gaussians to each reduction process and integrating the area under each curve, we estimate that 74.2% of the immobilized MB-DNA oligonucleotides have undergone toe-hold mediated strand displacement and are in the open conformation.

**Figure** 6. Electrochemical interrogation of the MB-DNA strand before and after toehold-mediated displacement. (a)-(b) After Step 1 (before the toehold-mediated displacement), a single reduction peak can be observed at a voltage of -0.5 V. (c) After Step 2 (after the toehold-mediated displacement), a reduction peak resulting from the combination of the reduction processes is observed at -0.55 V.

The shift in reduction potential of the open DNA hairpins arises from the sensitivity of the electrochemical properties of the MB label to the local environment (such as electrostatic potential, pH, solvent access) (21) which will be different depending on whether MB-DNA is in the open or closed conformation. While the electrochemical measurements are indicative of conformational change and can be used to quantify the efficiency of the displacement reaction, they do not provide detailed, unambiguous and quantifiable information about the structure of the DNA monolayer. For instance, non-specific binding of the strand Catalyst could restrict access of counter ions to the MB label, changing the thermodynamics of the electrochemical reaction. This means that the conformation of the DNA hairpin cannot be unambiguously inferred from the electrochemical measurements alone.

Using the electrophotonic approach, however, we can complement the electrochemical information with the precise structural information revealed by the cascaded ring photonic measurement. From the shifts in resonance wavelength following toe-hold mediated strand displacement (Table 2), we find that the thickness of the MB-DNA layer has only changed moderately, increasing from 1.97 nm to 2.21 nm. This increase is associated with a change in the length of the double stranded region (16 base pairs in the double stranded neck-region of the closed hairpin, compared to 23 base pairs following hybridization of Catalyst to MB-DNA, see SI). We also observe a significant increase in refractive index (from 1.427 to 1.443 RIU). As discussed above, the number of surface immobilized MB-DNA is identical before and after opening of the DNA hairpin. The change in refractive index is thus related to the length of the single stranded region attached to the surface. Single stranded DNA exhibits a short persistence length is thus highly conformationally flexible and dynamic (19). As a result, the open hairpin collapses onto the surface leading to an increase in refractive index (19).

Following an electrophotonic interrogation, we have therefore been able to precisely and unambiguously quantify changes in molecular conformation of MB-DNA from the optical data (orientation angle, *t* and *n*); while complementing it with information of the molecular layer assembly retrieved from the electrochemical domain (precise molecular surface density and efficiency of the displacement reaction).

CONCLUSIONS

We have introduced a novel configuration of electro-optical biosensors that is able to provide insight into complex conformational changes of surface bound molecular layers. Employing a single TE optical mode, quantification of the refractive index and thickness of molecular layers such as proteins and DNA oligonucleotides has been performed in real time by tailoring the light-matter interaction at the sensor surface. Our novel configuration not only reduces the complexity of the sensor design and experimental setup compared to dual-polarization approaches, but it can also be implemented on small footprints enabling a highly multiplexed sensor platform for parallel measurements. More importantly, the technique is unambiguous, as the single polarization used is guaranteed to probe the same molecular orientation, while TE and TM modes are oriented orthogonally and respond differently to changes in different directions. This approach can be further extended to a larger number of rings, which would ultimately reduce the noise of our system and further increase measurement accuracy. We have verified the technique against the well-characterized BSA protein system and have shown consistency with similar surface based techniques such as DPI and dual-polarization ring resonator analysis.The ability to combine with electrochemical interrogation not only further broadens the application-space, allowing multiparameter profiling of electroactive molecules such as enzymes or redox labelled molecules, but also provides deeper insight into the structure of molecular layers. Our technique thus provides a powerful analytical tool for quantifying and understanding of complex molecular and (bio)molecular processes.

ASSOCIATED CONTENT

Supporting Information

A PDF file is supplied providing information about the supporting simulations of the electrical field distribution at the sensor-solution interface, the modelling of the interaction optical field with the overlayer, thickness and refractive index accuracy calculation, simulations of the structural conformation of the DNA oligonucleotides employed, quantification of the MPTS-Cu molecular layer and the protocol to perform an in-line device geometry calibration.

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Recording the resonance shift from two geometrically distinct ring-resonators connected to a single access waveguide, unambiguous quantification of the thickness and the refractive index of a molecular layer assembled on the waveguide is obtained with a precision of the technique is 0.05 nm and 0.005 RIU in the molecular layer thickness and refractive index, respectively. This is further complemented with electrochemical interrogation to provide deeper insight into the biomolecular process occurring at the sensor surface. This novel sensor geometry provides a new approach for monitoring the conformation and conformational changes in an inexpensive and miniaturized platform that is amenable to multiplexed, high-throughput measurements.