

Protease detection using a porous silicon based Bloch surface wave optical biosensor

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Abstract: In this article we present an optical biosensor for label-free detection of trace levels of protease activity. The scheme is based on surface functionalized porous silicon optical structures which supports optical Bloch surface modes. The optical structure provides a resonant optical mode for high sensitivity detection and open access of the sensing layer to the target enzyme. Protease detection is based on the digestion of gelatin, covalently attached inside the pore space, resulting in a spectral blue-shift of the optical mode. Monitoring of spatially separated resonant optical modes is used to eliminate optical response from nonspecific adsorption.

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1. Introduction

Porous silicon (PSi) presents an almost ideal optical sensor platform, with potential in a diverse range of applications from trace environmental gas sensing to label-free monitoring of biomolecular interactions. The porous scaffold has an extensive internal surface area for adherence of analytes, which enables the modification of bulk properties of the porous layers, e.g. refractive index, through the use of surface related effects. The potential for sensing of biomolecular interactions using PSi was first realised by Lin *et al.* [1] who showed that simple single thin film interference could be used for affinity biosensing, where protein-ligand binding interactions could be detected with a pico-molar concentration detection limit. Since these early studies there have been two key developments which have greatly expanded the potential capability of this technology. Firstly, multilayered PSi optical films have been used to create high quality optical structures, such as rugate filters [2] and microcavities [3], which contain spectral features for improved sensitivity [4]. Secondly, a robust hydrosilylation chemistry [5] based on a highly stable Si-C bond has been developed to passivate the internal porous surface against oxidation. Such a surface chemistry approach may also be expanded to provide antifouling capabilities as well as receptors for selective binding of target molecules [6].

One perceived advantage of high finesse optical structures, such as optical microcavity resonators, in sensing applications arises from the improved resolvability of spectral shifts due to the sharp spectral features (e.g. cavity resonances). In PSi, using a large porosity modulation and optimised fabrication conditions, optical microcavities with resonant line-widths of 0.21 nm and Q-factors of up to 7000 have been achieved [7]. Part of the challenge of utilizing such high Q-factor microcavities for sensing, and particularly in biological systems, is that the low porosity, high refractive index, layers that make up the dielectric mirrors have small pore sizes (< 10 nm) and create a blocking layer which inhibits the diffusion of molecules to the cavity layer where the enhanced optical field is confined. This then restricts the degree of porosity modulation and consequently the overall finesse of the resonance mode available for sensing. Attempts to compensate for this by using optical structures with smaller porosity modulation and larger pore sizes in the mirrors lead to increased scattering losses, broader spectral features and increased mirror thicknesses [8].

More recently it has been suggested that optical Bloch surface modes formed at the interface between a periodic dielectric structure, such as a Bragg reflector, and an adjacent dielectric medium, could be used to overcome some of the challenges associated with high finesse structures. Bloch surface waves are characterised by an exponentially decaying electric field on either side of the dielectric - periodic structure interface, and in this way they are analogous to surface plasmon polaritons (SPP) [9]. One of the features which distinguish Bloch surface modes from SPPs is that they are supported between two dielectric interfaces rather than a metallic film making it possible to produce high finesse modes. In this sense Giorgis *et al.* recently showed Bloch surface waves supported by silicon nitride films could be used to detect refractive index changes as small as 3.8×10^{-6} [10]. Another important feature of the planar photonic architecture described above is that it is not restricted in terms of the open surface area available for sensing. This leads to the potential for large-scale label-free sensing of analytes, or with the introduction of patterning, micro-arraying and parallel screening of samples [11].

Optical Bloch surface waves have recently been realized in porous silicon photonic structures [12,13] and have been implemented for simple gas sensing applications [14]. However, the most significant advantage afforded by these structures is that the concentrated

optical mode located adjacent to the ambient medium is suitable for sensing large macromolecules, such as proteins and DNA. In this paper we present a first implementation of a porous silicon based Bloch surface sensor for the detection of protease activity. The sensing mechanism in this device is based on the digestion of a covalently attached gelatin substrate leading to a reduction of the refractive index of the porous films and a spectral blue-shift of the optical mode. This detection scheme has previously been successful in monitoring the release of Matrix Metalloproteinase (MMP) in small populations of cells in culture [15]. The surface functionalization also includes an antifouling layer to suppress non-specific adhesion of biomolecules to the porous matrix. The optical structure presented here provides a resonant optical mode for high sensitivity detection and open access of the sensing layer to exchange of enzymes and digestion products. Furthermore, we utilize monitoring of spatially separated resonant optical modes to eliminate optical response from nonspecific adsorption.

2. Sensor design and fabrication

The optical Bloch surface wave sensor used in this study is based on a periodic multilayered PSi Bragg reflector mounted on a glass substrate. The film is etched as a monolithic structure from highly boron-doped silicon wafers (resistivity: 1.5-2.0m Ω .cm, <100> oriented) in a 25% HF ethanolic solution at room temperature. Refractive index modulation is achieved by varying the current density to change the porosity of the films during etching. Porous silicon formation occurs as a self limiting electrochemical reaction between fluoride ions in the electrolyte and valence band holes from the crystalline silicon, at the interface between the bulk silicon and porous silicon film. The need for transport of ions to the reaction interface ensures that the porous network is continuously and intimately interconnected across the entire film. The pore size for a given porosity is well defined, with a pore-size distribution in the range of a few nanometers [16]. Using this method, high-quality photonic structures have been demonstrated and notable examples include high Q-factor microcavity resonators [3], broad reflectivity dielectric laser mirrors [7], and narrow band rugate filters [2].

The etching sequence used to fabricate the surface wave structures correspond to a Bragg reflector with alternately high (H) and low (L) porosity layers. Etching current densities and times used were 5 mA/cm² and 18.2 sec for the low porosity layers and 158 mA/cm² and 1.9 sec for the high porosity layers. Each layer was etched as a sequence of steps with breaks of a few seconds between steps to allow the electrolyte concentration at the reaction front to recover. This sequence was chosen to produce a broad high reflectivity band in the near-infrared spectral region for light incident at 45 degrees (through a prism coupler). The spectral region was chosen to correspond to the high sensitivity region of spectroscopy equipment used in the sensing studies. The etching time of the top high porosity layer was increased by a factor of 1.3 in order to position the surface wave near the centre of the high reflectivity band.

The dielectric structure is lifted off the native silicon substrate by applying a high current pulse in a 15% HF solution. Surface modification and sensor interface fabrication is undertaken in the procedure developed by Böcking *et al.* [17,18]. Briefly, the lifted-off film is functionalized with neat undecylenic acid through a hydrosilylation process to resist aggressive biochemical conditions and assist further modification. Next, the modified film is reacted with 1-amino-hexa(ethylene glycol) to form a layer that resists nonspecific adsorption of proteins on the surface *via* activation with *N*-hydrosuccinimide (NHS) and *N*-ethyl, *N'*-(3-(dimethylamino)propyl)-carbodiimide (EDC). Subsequently, the ethylene glycol terminated film is activated with *N,N'*-disuccinimidyl carbonate (DSC) and *N,N*-dimethylaminopyridine (DMAP), followed by covalent attachment of gelatin. The gelatin-modified device is cut off from the silicon substrate on the rim and placed on a No.2 glass coverslip.

The role of the gelatin terminated surface as a sensing element for protease activity has been previously established by this group [19]. The gelatin which fills the pore space of the porous silicon matrix acts as a substrate for target proteases. The catalysis of proteases, such as subtilisin or MMP, causes hydrolysis of peptide bonds in the gelatin molecular chain, which leads to a reduction in the refractive index of PSi as the gelatin network is broken down

and is replaced with air. Such a change in the refractive index of the top layer is embodied by a spectral blue shift of the surface wave mode.

3. Experimental procedure

Optical measurements were performed on a prism coupling arrangement as depicted in Fig. 1(a). In this set-up a fiber-coupled white light source is collimated to a diameter of 10 mm and then passed through a polarizing beam splitting cube to produce an incident beam with TE-oriented polarization. The light is then focused onto the internal reflecting surface of a right-angle prism (BK7) using a 10 cm focal length lens; a mirror is used to redirect the beam such that the incident angle is 45° . The total footprint of the incident beam on the sample surface is of the order of 100 μm . Light reflected from the sample is collected by a second lens and coupled to an Ocean Optics USB2000 + spectrometer using a fiber coupler. Figure 1(b) shows a detailed diagram of the sample mounted on the prism surface. The glass coverslip with porous silicon multilayered structure is mounted on the top surface of the prism using an appropriate refractive index matching liquid. A blank coverslip is used to provide a reference spectrum that can be used to correct for the spectral sensitivity of the system.

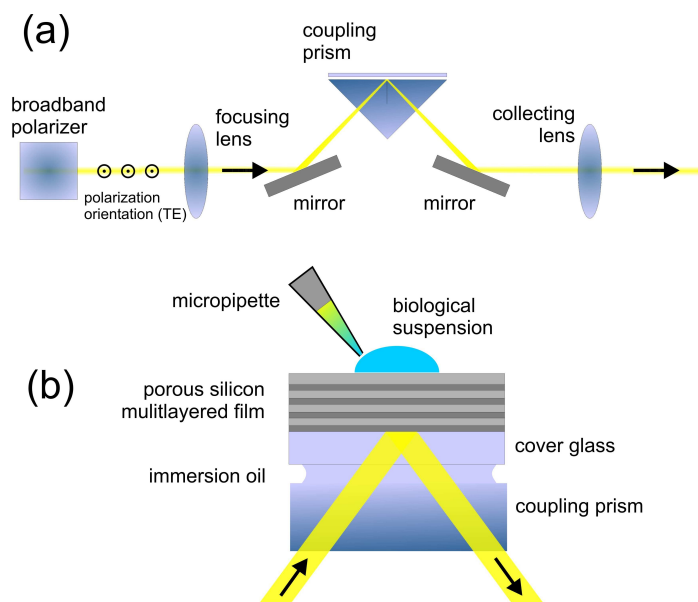


Fig. 1. a) Diagram of the Kretschmann-type prism coupling optical arrangement used for measuring the reflectivity of the surface wave sensor. The incident white light was polarized in the TE orientation and focused onto the prism using a 10 cm focal length lens. The reflected light is collected by a second lens and coupled to a spectrometer. b) Detailed diagram of the porous silicon film mounted on the surface of the prism using a cover glass as a substrate and refractive index matching liquid. During the sensing experiments the biological suspension is applied to the top surface and permeates through the porous film. After a fixed time the solution is removed from the film and allowed to dry.

Enzyme assays were assessed by applying 5 μL aliquots of different solutions of biological media to the exposed surface of the photonic structure using a micropipette. The initial reflectivity from the sample was measured and then the solution was applied and left on the sensor surface for a fixed time of 20 minutes, before being removed using the pipette. During the exposure the porous silicon structure was maintained in humid conditions to avoid drying. The reflectivity was then measured again and the sample was spotted with Milli-Q water to remove any buffer salt residue deposited on the surface. For control tests a biological phosphate buffer saline (PBS) solution was used, containing NaCl 137 mmol/L, KCl 2.7 mmol/L, Na_2HPO_4 10 mmol/l, KH_2PO_4 1.76 mmol/L. For the enzyme test 0.01 mg/mL of

subtilisin, a broad-based specificity protease, in PBS, was applied to surface wave sensor; this corresponded to 1.8 pmol of subtilisin.

The amount of gelatin attached in the pores is in excess of the enzyme applied, so it meets the criterion of Michaelis-Menten kinetics, i.e. the reaction is of zero order, where the initial reaction velocity is proportional to the concentration of enzyme. At the initial stage of the reaction, depletion of gelatin, represented by the surface mode shift, is a measure of the velocity and is thus linearly dependent on the enzyme concentration. The linear relationship between initial velocity and enzyme concentration is also the basis of enzyme (activity) quantification.

4. Results and discussion

Figure 2(a) shows the experimentally measured (solid) reflectivity spectrum for a freshly etched *PSi* surface optical Bloch surface wave sensor showing all the important spectral features. The resonance at 632 nm corresponds to the surface wave mode, which the incident light is coupled by frustrated total internal reflection through the truncated Bragg reflector. The spectral band (489–807 nm) in the region around the surface wave mode indicates the reflectivity stop band of the dielectric mirror. Light incident on the *PSi* films will experience strong Bragg reflection at these wavelengths. The reflectivity dips at the band edges of the stop-band relates to increased absorption/scattering within the films due to Brillouin-scattering type slow light modes. At the longer wavelengths the light is reflected at the *PSi* / air interface by total internal reflection and is close to 100%. At shorter wavelengths the incident light is absorbed strongly by the silicon component of the porous silicon films and the reflectivity is modified accordingly. Importantly, if the multilayer structure exhibited very low scattering/absorption losses, light coupled into the band-edge and Bloch surface modes would be out-coupled in the same direction as the reflected light and this would reduce amplitude of the resonance dips observed in reflectivity. This means that the intrinsic losses in the porous silicon films aid in resolving the spectral positions of the different modes in photonic structure. The importance of the TE polarization is understood when considering Fresnel reflection of each of the dielectric interfaces at non-normal incidence. For TE polarization the reflectivity increases monotonically with increasing angle of incidence, whilst TM polarized light the reflectivity decreases toward zero at Brewster's angle. This results in a TM reflectivity band with a smaller spectral width and lower absolute reflectivity, which are unsuitable for supporting surface modes

Modeling of the *PSi* multilayered films is done using the matrix transfer method. The refractive index of each of the layers is estimated using a mean effective medium Bruggeman model, taking into accounts the relative fraction of crystalline silicon and voids in each layer. This model also accounts for dispersion and absorption of silicon component of the *PSi* films at the short wavelength part of the visible spectrum. The model does not account for attenuation due to scattering. A three part Bruggeman model [20] is used to simulate the effect of the surface modification due to surface derivatization and the activity of the enzyme. In this case a fraction of the pore space of the *PSi* is filled with materials of different refractive index, thereby displacing the lower refractive index air. A simulation of the reflectivity spectrum (dotted) for this structure is also depicted in Fig. 2(a), which shows good correspondence with the measured data. From the simulation we determine that the high refractive index layer has porosity of 44% and a thickness of 71 nm and the high porosity film has a porosity of 75% and a thickness of 129.5nm. The top high porosity layer is 1.3 times the thickness of the other layer and is used to tune the position of the surface wave mode relative to the band edge [13]. A plot of the corresponding refractive index profile of the photonic structure for the surface wave mode wavelength is shown in Fig. 2(b). We note a small discrepancy between the simulated and measured positions of the long wavelength band-edge position.

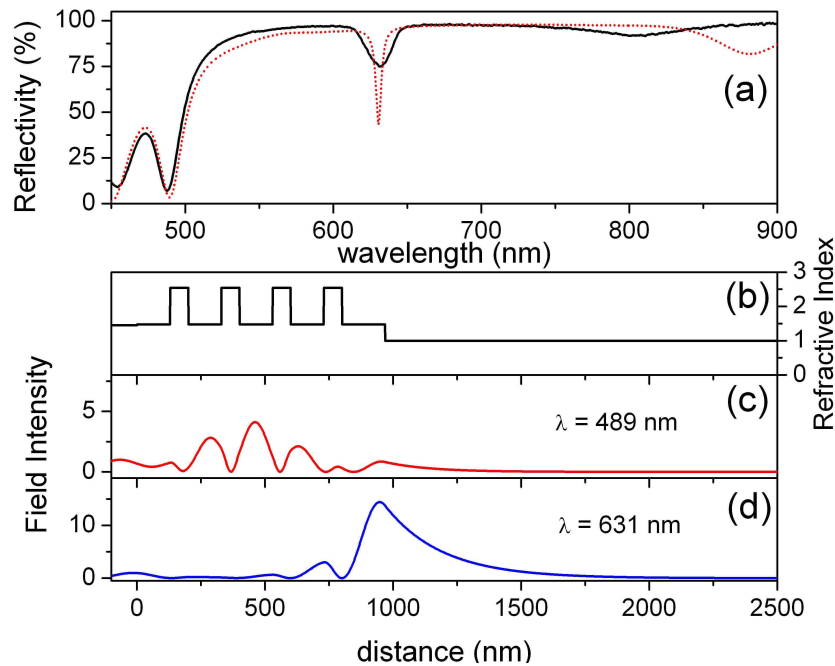


Fig. 2. (a) Experimentally measured (solid) and simulated (dotted) reflectivity spectrum for a freshly etched porous silicon optical Bloch surface wave sensor. Important features include the surface wave mode (631 nm), and the two band-edge modes (489 – 807 nm). (b) refractive index profile of the surface wave structure at the surface wave mode coupling wavelength. The distance is measured relative to the interface between the prism and the multilayered film. (c)-(d) Field intensity profiles for wavelengths corresponding to the band-edge modes (c) and the surface wave mode (d).

Figure 2(c), 2(d) shows the simulated electric field intensity for different modes within the photonic structure. The distance is made relative to the interface between the prism and the multilayered film. These include: the short wavelength band-edge mode at 489 nm (Fig. 2c), and the surface wave mode at 631 nm (Fig. 2d). At other wavelengths the field intensity decays exponentially within the structure. The band-edge mode exhibits a five-fold enhancement of the field intensity with respect to the incident beam, whilst the surface wave mode shows a fifteen-fold enhancement.

Figure 3(a) shows the measured reflectivity spectrum of a surface-modified surface wave sensor before (solid) and after (dotted) exposure to the enzyme subtilisin. The initial curve corresponds to a surface functionalized photonic structure with the gelatin network filling approximately 30% of the pore space in the top layer of the Bragg structures. Upon application of the solution we observe a blue shift of 8 nm in the resonance mode and a 15 nm red shift in the band-edge mode. The action of the protease on the optical sensor is expected to result in a blue shift of the surface mode due to the digestion of gelatin on the surface. The red shift in the band-edge mode we associate with non-specific adsorption of buffer salts residue and byproducts of the enzymatic reaction deposited throughout the entire photonic structure. Upon rinsing with deionized water and drying (Fig. 3(b)) we observe that the band-edge mode shifts back to the origin position and the surface wave mode is further blue shifted to 31 nm with respect to the initial position. This suggests that the optical shift due to non-specific adsorption acts to reduce the sensitivity of the enzyme detection, however simple rinsing recovers the full mode shift. Figure 3(c) shows the evolution of the blue shift in the Bloch mode position as a function of time for a sensor exposed to a solution containing 0.007 mg/mL of subtilisin. This is done by removing the enzyme to stop the reaction at fixed time intervals and measuring the resultant shift. From this graph we see that a significant blue shift

in the mode position is observable after only two minutes of exposure. The reaction kinetics presents a measure of the substrate (gelatin) concentration on the sensor surface. For a separate control test where the sensor is treated with only biological buffer, both the band edge and the surface mode recover their original position after rinsing. This is also seen in the kinetics where the control test yields no net shift in the peak position.

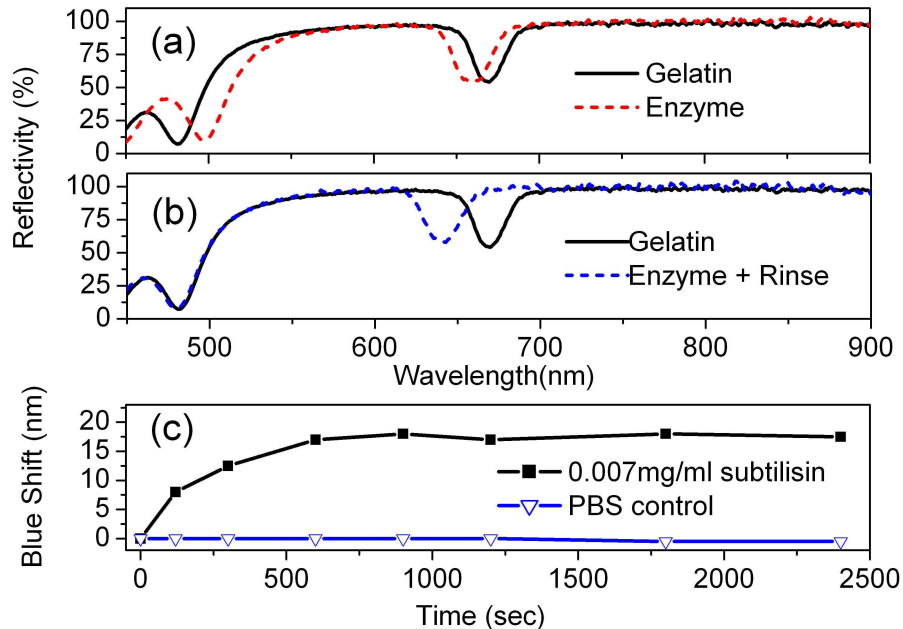


Fig. 3. (a) Experimentally measured reflectivity spectra of surface functionalized porous silicon optical surface wave sensor before and after exposure to 0.01mg/mL of enzyme in biological buffer. (b) Reflectivity spectra after the structure in (a) has been rinsed in deionized water. (c) Graph showing the evolution of the blue shift in the Bloch surface mode (relative to the band-edge mode) when exposed to a solution containing 0.007 mg/mL of Subtilisin. The graph also shows the response of the sensor to a blank test for biological buffer.

Importantly, these results show that porous silicon optical structures supporting resonantly coupled optical Bloch surface waves may be used for the detection of enzyme activity. The unique geometry of this structure with the sensing element located at the interface between the photonic structure and the ambient medium allows enzymes to freely migrate to the sensing element and act on the gelatin. This is in contrast with other resonant structures, such as a microcavity, where the sensing element is buried under a multiple layers of porous silicon, which effectively block the mass transport of enzymes to and digestion products away from the sensing region. Compared with single layer and rugate filter type optical structures, the narrower spectral features of the resonant modes allows for improved resolution of spectral shifts and leads to the possibility of lower detection limits.

Another important feature of the optical structure presented in Fig. 3 is that the band-edge mode is not sensitive to changes in the optical properties of the top layer. This provides a means of discriminating between refractive index changes occurring throughout the photonic structure, resulting from non-specific adsorption on the porous silicon internal surface, and enzyme specific refractive index changes that are confined to the top surface. The relative sensitivity of the band-edge and surface modes to refractive index changes in the sensing layer may be understood by considering the spatial distribution optical mode, presented in Fig. 2(c) and (d). The surface wave has strong modal confinement at the interface between the Bragg reflector and the ambient medium. The spectral position of the mode will therefore be highly susceptible to changes in the optical thickness of the top layer. In contrast the band-edge field

intensity is distributed through the bulk of the structure and only a small component of the mode overlaps with the top layer, and will therefore be only weakly dependent on the changes to the top layer.

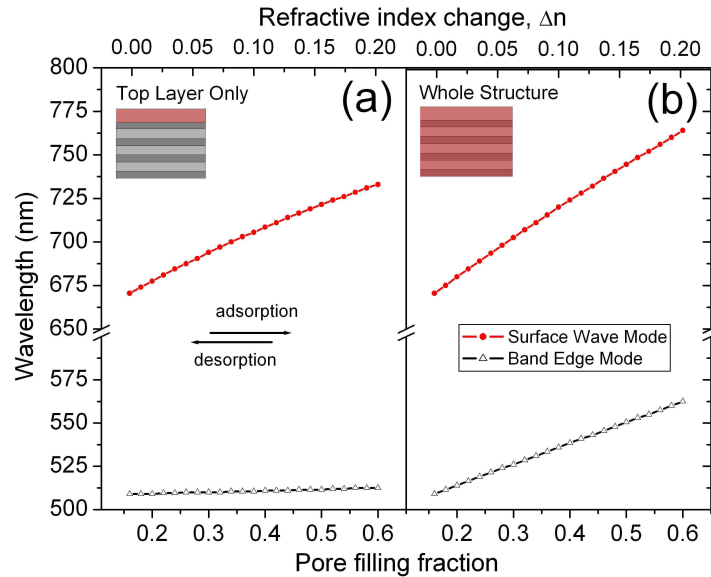


Fig. 4. The predicted spectral response of the optical Bloch surface mode and Band-edge mode to changes in the relative fraction of gelatin filling the pore space in the of the porous silicon layers: (a) changes occurring only in the top layer, and (b), changes occurring uniformly through the entire structure. The corresponding change in refractive index is displayed on the top axis. The band edge mode has a weak dependence on changes to the top layer and this may be used to discriminate between optical shift arising from specific and non-specific adsorption.

Figure 4 shows a simulation of the change in the spectral position of the band edge and surface wave modes when there is a change in the refractive index (a) in the top layer and (b) uniformly throughout the structure. The simulation is based on changing the relative fraction of gelatin ($n = 1.45$) inside the pore space ($n = 1.0$) of the porous silicon and uses the three part Bruggeman model to determine the corresponding refractive index change. The wavelength shifts are shown with respect to a pore filling fraction of 0.16, which corresponds to surface passivation and antifouling layers. In Fig. 4(a) we see clear distinction between the flat response of band-edge mode compared with the surface wave mode ($d\lambda/dn \sim 295 \text{ nm / RIU}$). Figure 4(b) shows that both modes give an approximately linear response when the refractive index change occurs throughout the entire structure. These results suggest that the spectral shift of the surface wave mode due to enzyme activity may be determined in the presence of a strong shift due to non-specific attachment by simply taking into account the shift of the band-edge mode. For example, a 15 nm red shift in band-edge mode observed in Fig. 3(a) is associated with 27 nm red shift in the surface wave mode. Taking this into account in the observed 8 nm net blue shift we should expect that the enzyme-related shift should be a 35 nm blue shift. This corresponds reasonably to the 31 nm blue shift observed in surface mode position after rinsing. These results suggest that by making a differential measurement we may measure a targeted response in the presence of a much larger background drift in the optical spectrum. Note that this is a unique feature of porous silicon based sensors where the entire photonic structure is permeable to the sample solution, allowing the band-edge mode to be accessible.

In conclusion, we have demonstrated detection of protease activity using a porous silicon Bloch surface wave optical sensor. The optical structure presented provides a resonant optical mode for high sensitivity detection and open access of the sensing layer to exchange of enzymes and digestion products. Monitoring of the spectral shifts with respect to the band

edge enables us to eliminate optical response from nonspecific refractive index changes that occur over the entire structure. We envisage considerable scope for this optical sensing platform in monitoring a range of biomolecular interactions.