Detection of bioanalyte using porous silicon based optical biosensor

V. MISHRA*, P. N. PATEL, A. SHARMA,T. DINESH KUMAR, V. GARG, T. BAFNA, D. MAHESHWAR
Dept. of Electronics Engineering, S. V. National Institute of Technology, Surat, India

Porous silicon has been confirmed as an active support for immobilized protein binding. Porous silicon has advantages of an extremely large internal surface area and the one of the most important being label-free. As compared to monolayer techniques this technique enables immobilization of large amounts of receptor. This paper is concerned with detection of bioanalyte using porous silicon based biosensor to detection of a very small concentration of bioanalyte avidine. Charactristic studies are carried out in fourier transform infrared spectroscopy (FTIR) in which infrared spectrum shows that bonding between the bioanalyte system and biosensor.

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Keywords: Porous silicon, Bioanalyte- Avidin , FTIR spectrum

1. Introduction

Optical biosensor works on change in refractive index of porous silicon due to different analytes. This change is due to different binding affinity of different analytes or biomolecules towards Porous Silicon (PSi) such as DNA hybridization or antigen-antibody binding[1]. Porous Silicon (PSi) has become a very good option for bio sensing applications due to its large surface area to volume ratio, increasing the possibility to immobilize more number of bioanalytes and which considerably enhance the possibility of detecting target analytes[2]. The physical properties of PSi depend directly on its structure and the optical properties are associated with change in refractive index due to variation of porosity while the electrochemical properties depend on surface modification. The surface of PSi is basically regulates Si-H bond's high reactivity, -H can be replaced by –alkyl or –OH. Thus, the surface of PSi can be made either hydrophobic or hydrophilic, and an enormous number of bioanalytes can be immobilized. In this paper, a PSi based biosensor which has the ability to detect biological molecule - Biotin and protein – Avidin is discussed. FTIR spectroscopy is used to carry out the characteristic studies [3, 4] Avidin is a protein found in the eggs of birds, amphibians, and reptiles. It is produced in the oviducts of these animals. An oviduct is the route leading from the ovaries to the outside of the body in non-mammalian vertebrates. In view of 100% molecular weight of Avidin only 10% weight is imputed to contents of Carbohydrate which is further divided into four to five mannose and three N-acetylglucosamine residues. Three or more structural types of oligosaccharide are present in Avidin which is alike in structure and configuration. In different areas of biology, the biotin-binding protein Avidin plays an important role due to its numerous characteristics like highest affinity (Ka~10^{15} M^{-1}) between a ligand and a protein. Avidin is basically a glycoprotein contained both mannose and N-acetylglucosamine. . Nonglycosylated avidin has essentially the same biotin-binding characteristics as glycosylated avidin and can be isolated from crude avidin preparations (~30% of total) by using concanavalin-A columns which has affinity to adsorb glycosylated part only. As an alternative, enzymatic cleavage is used for deglycosylation. Deglycosylated Avidin are expected to have lower nonspecific binding. The purification of avidin is done by using the process of adsorption on an insoluble biotinylated matrix but due to its high affinity with biotin it is not eluted easily.

2. Materials and methods

Fabrication of PSi by the method of electrochemical etching is the first and basic step. Before the silicon film is etched using this process, it is cleaned for any impurities on its surface. Piranha cleaning method is used to clean organic residues off substrates. Piranha solution is a mixture of Sulphuric acid and Hydrogen Peroxide in 3:1 ratio. Piranha solution being a strong oxidizing agent, removes most of the organic matter and hydroxylates surfaces, making them hydrophilic[5]. Fabrication was done using the electrochemical etching cell. PSi is known to form during electrochemical dissolution of silicon in HF-based solutions. This dissolution is obtained by monitoring either the anodic current or potential. PSi structures are fabricated by electrochemical etching of p-type Si wafer by adjusting the anodization current density at constant electrolytes concentration and etching time. The morphology of the pores of PSi is used to explore properties like shape, evenness, interconnections and orientations etc.

The parameters that affect the formation of pores on silicon surface during fabrication process are Substrate doping, Electrolyte concentration, Solvent in which Electrolyte is dissolved, Current density, Etching time etc PSi's optical constants depend on surface porosity, on the relative air content of the silicon-air mixture. Thus, by varying the porosity value of PSi its different refractive indices can be obtained. Single layer interferometer is a simplest PSi optical sensing device. As shown in Fig. 3 light traveling from one medium with refractive index n_0 encounters a thin film with refractive index n. Some of the light is transmitted through the film and some other is reflected. For the case of PSi layers, the incident medium is air, its refractive index being n_0=1. The reflectivity spectrum of the film is described by the multiple interference fringes caused by the air-porous silicon and porous silicon-silicon interfaces.

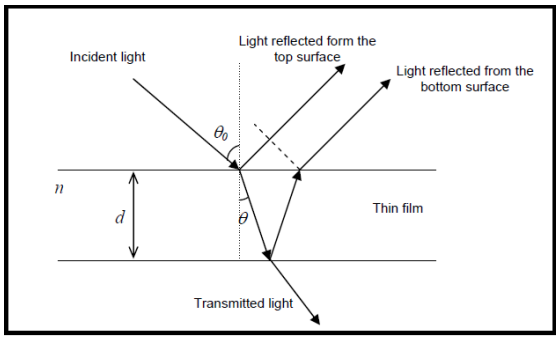

Fig. 3. Light incident on a single layer of porous silicon

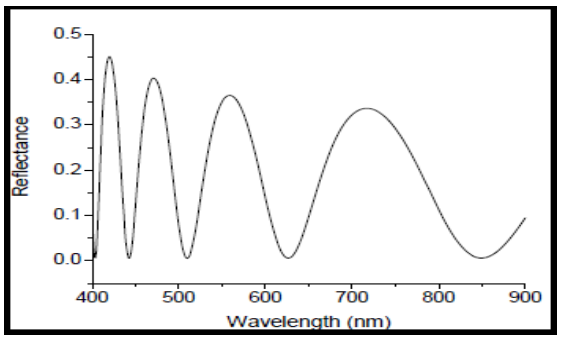

Fig. 4. Light reflectance pattern observed for a single layer etched porous silicon

Due to constructive or destructive interference at various incident wavelengths, an interference fringe pattern is obtained, such as the one shown in Fig4. Biomolecular binding events inside the PSi increase the effective refractive index of the PSi layer and interference pattern is shifted towards the longer wavelength. Distributed Bragg reflectors (DBR) are dielectric multilayered structure based on the effect of multiple interferences that occur when a light beam is reflected by various dielectric interfaces. DBR are multilayered structures formed by the periodic repetition of two layers with different refractive index (n_H and n_L) and thicknesses (h_H and h_L, respectively). Figure 5shows a high reflectivity, centered at wavelength λof a DBR reflectivity spectrum due to the fact that for the wavelengths close to this λ, beams, reached at surface interface have same phase after being reflected by the several interfaces, Thus they interfere constructively.

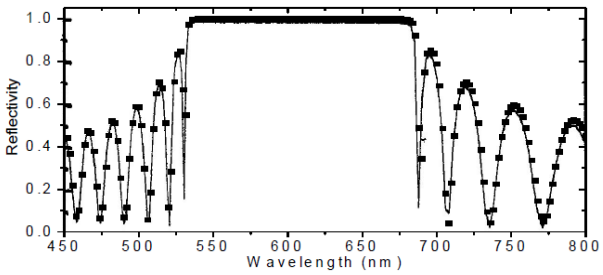

Fig. 5. Reflectivity of a DBR with n_H=1.86 and n_L=1.27 Repeated 20 Times and λ= 600 nm Light

The most commonly used structure of DBR is the quarter wave DBR, where the optical thickness of the layers is $n_Hh_H = n_Lh_L = \lambda/4$ and the period of the structure is $\Lambda = h_H + h_L$. The schematic structure of a DBR is shown in Fig. 5. In order to achieve better sensitivity in a PSi optical structure for sensing applications, PSi microcavities have been fabricated. A microcavity structure is a multilayer structure in which there is a defect layer between two Bragg reflectors. Microcavities are band-pass filters, peak position is tuned, tunable mirrors and detectors of substances like ethanol, methanol, acetone, chlorobenzene and nitrogen dioxide. They also improve the light emitting capacity of PSi.

Ideally Avidin has 3D architectures as biotin-binding pairs are arranged on opposite sides of the molecule, leading to defined molecular networks. A wider range response can be obtained by varying number of layers used for sensing of avidin, biotinylated enzyme and their molar ratio. The avidin-biotin system is used in immunoassay in two main categories. i.e. firstly, insertion of avidin in the immunoassay protocol to intermediate between probe and antibody or antigen and secondly, its immobilization onto the solid phase for improving its characteristics of the capture system. The extremely high affinity constant ($Ka \sim 10^{15}$ M^{-1}) and specificity of avidin for the vitamin biotin are the main reasons for use of the avidin-biotin system. In addition, avidin's occurrence as a tetramer in solution, i.e. it has four biotin-binding sites per molecule. In immunoassay systems it is particularly useful because the tetrameric structure is one of the main reasons for amplification of the signal. The other reason is covalently attachment of many copies of the biotin moiety to any protein molecule virtually. Multiple copies of biotin on an antibody or protein can then interact non covalently, but very strongly, with the four binding sites of avidin. This strong non covalent cross linking among the components results in an enhancement of the primary interaction[6]. The conjugation of avidin- biotin methods have more benefits as they are independent to isoelectric point (pI) of protein, needs less quantities of biotinlayted

legend, usage of bioltinylation kits which are easy to use, availability of huge variety of boltinylated reagents, single injection is required for the immobilization process their precise control over concentration of bound conjugate immobilized by dismissing the injection when the preferred Rmax, maximum reaction, is touched, their surfaces possess low electrostatic charges, and a single biotinylation reaction usually yields enough product to allow an almost unlimited number of immobilizations. For characterization we have to stick biotin on PSi surface but biotin will not stick directly on PSi surface so that we have to do some treatment in line like- APTES treatment and Glutaraldehyde treatment than biotin will stick on the surface easily which will use for detection of avidin protein. In APTES which is 3-Amino propyl tri ethoxy silane has three C-N bonds and nine C-N bonds, in a fully polymerized molecule and in an adsorbed, unhydrolyzed, unpolymerized molecule respectively.

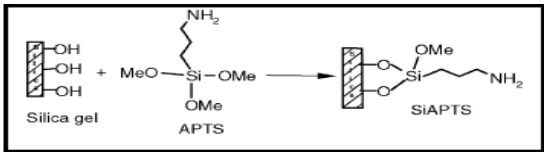

Fig. 6: Reaction of APTES with Etched Silicon to Give Organo-Functionalized Silica with N-H and OSi-C Bonds

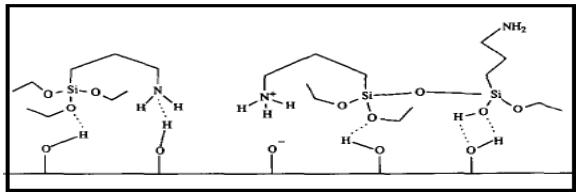

Fig. 7: Binding of APTES to Silicon Surface

Porous silicon substrate was immersed in a 2 % APTES solution and alcoholic mixture with DI Water and methanol (1:1) solution for 20 minutes at room temperature. Then, substrate was removed from the APTES solution and were rinsed in DI water. Substrate was dried in inert Nitrogen atmosphere and heated at 100°C over 10 minutes and the solvent vapors were evacuated. By adding the APTES the peak positions of substrate silicon and oxygen do not change as the monolayers of APTES are positioned with free amine groups which are away from surface of silicon and with hydrogen-bonded and close to surface are protonated amines are than bind to silicon oxide.

The incubation of silicon in APTES solution leads to formation of thick layers. These layers are soluble in water as they are held together by weak forces and weak bonds with hydrogen. The APTES is first adsorbed non-covalently by silicon.

Homo-bifunctional reagents on reaction with primary amine groups exhibit various characteristics as they can form stable inter-intra-subunit covalent bonds which are soluble in aqueous solvents and are widely used. In many applications Glutaraldehyde a popular reagent is used where protein's structural rigidity is necessary, like in this case of protein interaction and detection.

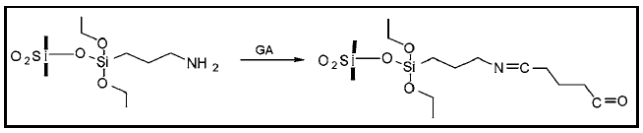

Fig. 8: APTES Treated Porous Silicon Reacts with Activator and Cross Linker – Glutaraldehyde

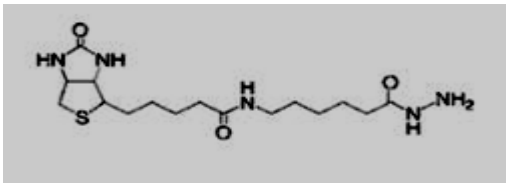

Fig. 9: Structure of Biotin Hydrazide

Advantages of using Glutaraldehyde as a cross linker between the inorganic Silicon chip and the organic biomolecules is that Glutaraldehyde is a smaller molecule (0.8 nm) resulting in a larger number of probe molecules immobilized in the pores. Also, it forms a thinner monolayer inside pores and leaves larger space for biomolecular immobilization.

Biotin – avidin system requires biotin to be attached for the detection of avidin. The hydrazide derivatives of biotin are mostly reacted with carbohydrate portion of glutaraldehyde. This method for biotinylation of polyclonal antibodies is an ideal due to heavily Glycosylation. The reaction with hydrazide derivatives of biotin is affected by Temperature, pH of oxidation and the periodate concentration. As glycosylation is varied with each protein, so optimum conditions must be determined and maintained for each glycoprotein.

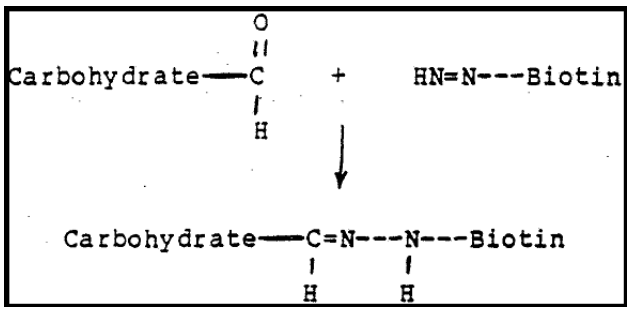

Fig. 10: Binding of APTES to Silicon Surface

The pH condition is optimum for oxidation and for the hydrazide-mediated biotinylation for preparation of glycoprotein. It is not recommended to use Tris, or other primary amine-containing buffers in both the oxidation and biotinylation steps because in these steps reaction of buffers with aldehydes and hydrazides's quenching of the

reaction is done. The spacer arm in Biotin Hydrazide allows optimal accessibility of avidin conjugates to biotin.

3. Results and discussions

Fig. 1 shows the FTIR Image of The Freshly Prepared Porous Silicon Microcavity Structure. In FTIR Image of The Freshly Prepared PSi Microcavity Structure, For Si-Si bond, a peak at 536 cm^{-1}confirmed the stretch vibration mode, as observed in the FTIR results of etched PSi. For Si-H bonding, PSi microcavity stage showed peaks at 2112 cm^{-1} to indicate stretch vibration mode of the Si-H bond, 914 cm^{-1}, 623 cm^{-1},and 2081 cm^{-1} corresponding to SiH_2 bond with the latter one for stretch vibration mode and 2138 cm-1 for stretch vibration mode of SiH_3 bond. High absorbance at 2050 cm-1 shows presence of SiH_x bond.The thickness of PSi can be determined from cross sectional pictures taken by a scanning electron microscope (SEM). For microporous silicon, the SEM image of the cross section of a PSi layer shows the porous zone clearly distinguished from the substrate. For this reason it has been used for the thickness estimation of our monolayers. SEM images present different gray planes depending on the porosity of silicon wafer layers. For this reason, the thickness of the different layers of a multilayer can be measured. The SEM used for the experiment was a Field Emission Gun-Scanning Electron Microscope with the model number JSM-7600F. Fig 2 shows the layers obtained and their thicknesses, using SEM.

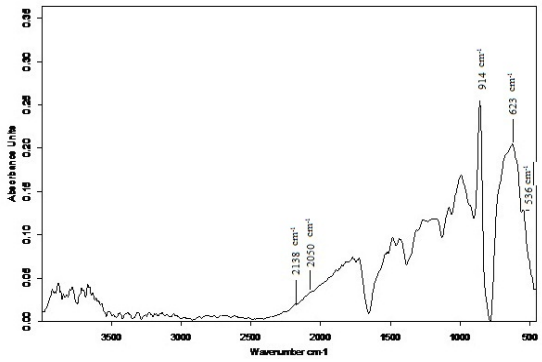

Fig. 1.FTIR Image of Porous Silicon Microcavity Structure

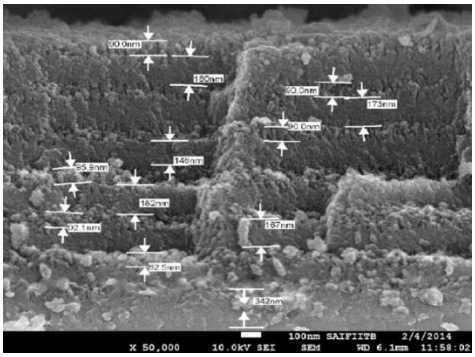

Fig. 2. Layers 1-13 of The 25 Layer Microcavity Shown Using FEG-SEM.

$$n_c d_c = \lambda / 2 \quad (1)$$

We can obtain the layer refractive index using Eq 1 where n_c is refractive index of the cavity layer and d_c is the thickness of the cavity layer. The factors that control the porosity of a PSi layer include the substrate doping, anodization current density, the concentration of HF and the duration of anodization. The porosity of a layer increases with increase in current density, with decreasing HF concentrations, increasing pH values of the electrolyte and slightly with increase in etching time. For our multilayer structures, very thin layers of PSi are formed. As previously stated, the porosity cannot be obtained directly on these thin layers. We can calculate the porosity of PSi by using Bruggeman Effective Medium Approximation (BEMA).

$$(1\text{-}P)*(\varepsilon_{Si} - \varepsilon_{PSi})/(\varepsilon_{Si} + 2\varepsilon_{PSi}) + P*(\varepsilon_{air} - \varepsilon_{PSi})/(\varepsilon_{air} + 2\varepsilon_{PSi}) = 0 \quad (2)$$

where, $\varepsilon_{Si,}$ $\varepsilon_{PSi,}$ ε_{air} represent the dielectric function of silicon, PSi, air and P is the porosity of the PSi. Also,

$$\varepsilon = n^2 \quad (3)$$

Where, n represents refractive index.

Another characteristic of porous silicon to be determined is Q-factor. The Q-factor of a microcavity is used to evaluate confinement of light within a microcavity structure of PSi.

The improvement in photo-stability of PSi is one of the key concerns of visibly luminescent PSi films, so number of treatments have been developed for e.g. electrochemical oxidation using KNO_3-H_2O, chemical oxidation using boiling water or HNO_3 and H_2O_2, photochemical oxidation with UV-DI water, plasma assisted oxidation with either H or O plasma, and thermal oxidation at high temperatures and flowing oxygen gas[7]

In the reflection spectrum of APTES treated silicon sample, There is a shift of 20.03 nm observed in the oxidized silicon sample treated with APTES to give the wavelength of 827.02 nm owing to the refractive index of 1.462[8].

The FTIR analysis of the APTES treated samples was carried out, with emphasis on the C-H, N-H, C=O and Si-NH2 bonds, to give the following results. As per the product of the reaction, primary amine is indicated in the FTIR images by two bands between 3100 $cm^{-1}$and 3500 $cm^{-1}$and one between 3700 cm^{-1} and 3900 cm^{-1}.

Three peaks at 3180 cm^{-1}, 3500 $cm^{-1}$and 3788 cm^{-1}confirm this theory. NH2 group is further confirmed with a peak at 1594 cm^{-1}. Methyl groups are confirmed of their presence by peaks at 3156 cm^{-1}, 2951 cm^{-1} for antisymmetric stretch mode of sp3 carbon atom and at 2875 cm^{-1} for symmetric stretch mode of sp3 carbon atom. The peak at 799 cm^{-1} is designated to the sp2 carbon atom.

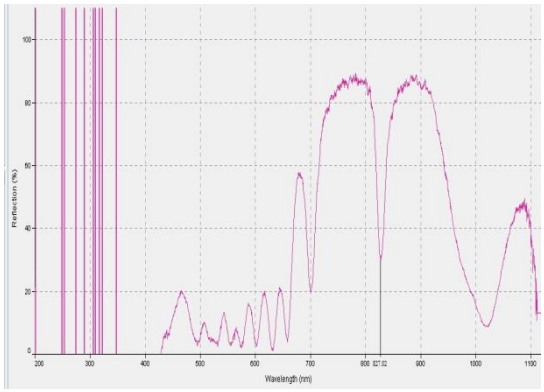

Fig. 11. Reflection spectrum of APTES treated silicon sample

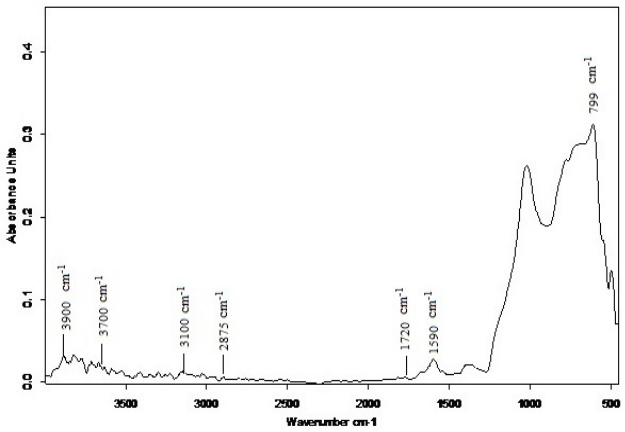

Fig. 12. FTIR spectra of APTES treated sample of porous silicon

In the reflection spectrum of Glutaraldehyde treated sample, The reflectance was observed to have undergone destructive interference to give the minimum reflectance at 852.19 nm and thereby a wavelength shift of 25.17 nm due to the refractive index of 1.348.

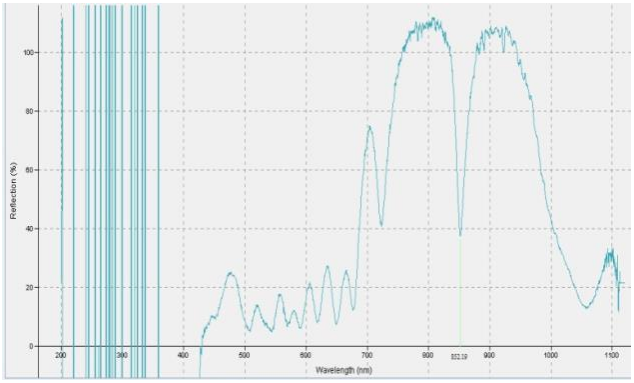

Fig. 13: Reflection spectrum when APTES treated sample is treated with crosslinking reagent glutaraldehyde

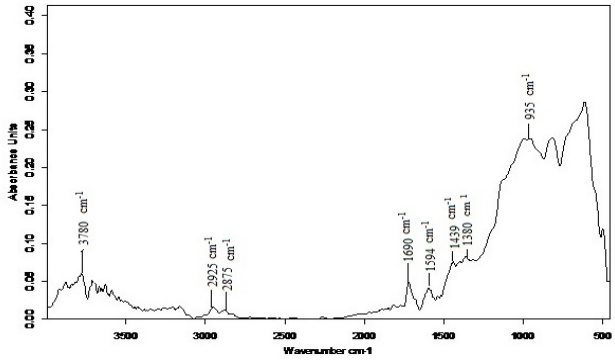

Fig. 14: FTIR spectrum of glutaraldehyde reacted sample

FTIR results of the GAA treated samples were studied for the C=N, C=O bonds and methyl groups. C=O group was indicated by the peak at 1719 $cm^{-1}$to denote the stretch vibration mode. Peak at 3780 cm^{-1}marks the presence of C-OH group, the state in which C=O group is also known to exist. C=N bond, as seen in the product is denoted by the peak at 1690 cm^{-1}. C-H bonds are present, as indicated by the peaks at 2875 $cm^{-1}$and 2925 cm^{-1}, where peak at 935 cm^{-1} points to CH_3 group. The highly reactive C=N bond is also known to exist in a different form- as C-NO_2 group. This is evident from the peaks at 1380 cm^{-1}, 1439 cm^{-1} and 1594 cm^{-1}. When comparing the carbonyl group (C=O) in APTES treated and GAA treated, the absorbance is higher in the latter.

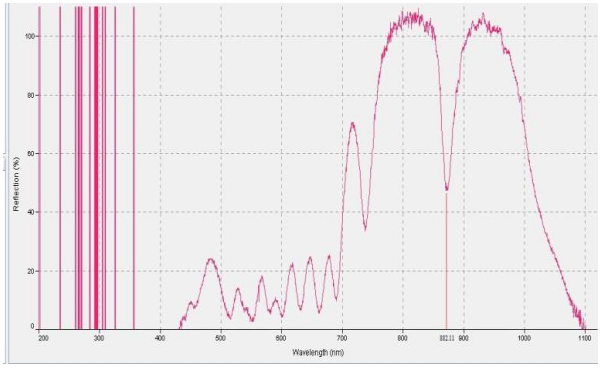

Fig. 15: Reflection spectrum of biotin treated silicon sample

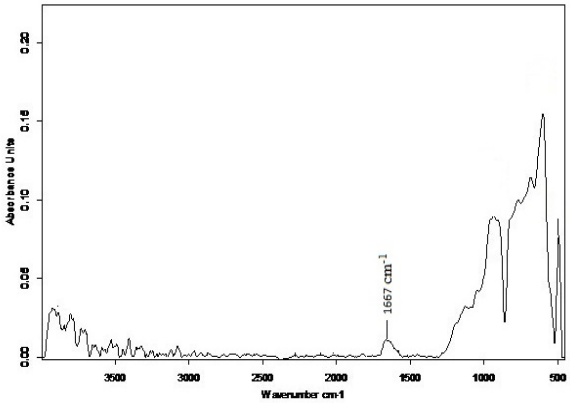

Fig. 16: FTIR absorbance spectrum for biotin incubated silicon sample.

In the reflection spectrum of Biotin Hydrazide treated sample, The reflectance was observed to have a minimum transmittance at 882.11 nm as biotin hydrazide has a refractive index of 1.544. The wavelength shift was observed to be 29.92 nm. FTIR results at 1667 cm^{-1} has been observed and documented to be the peak that indicates the carbonyl bond of reaction with biotin.

To demonstrate the biosensing ability of the fabricated and functionalized PSi, we have detected Avidin of different concentrations (20μM, 16μM, 14μM, 12μM, 10μM) using the technique of refractive index based transduction. These 5 observations have been used to detect presence of pure analyte using the biosensor functionalized with the protein biotin, towards which it has an extremely high affinity coefficient. Fig 17 shows the spectrum of biotin and very less concentrated avidin.

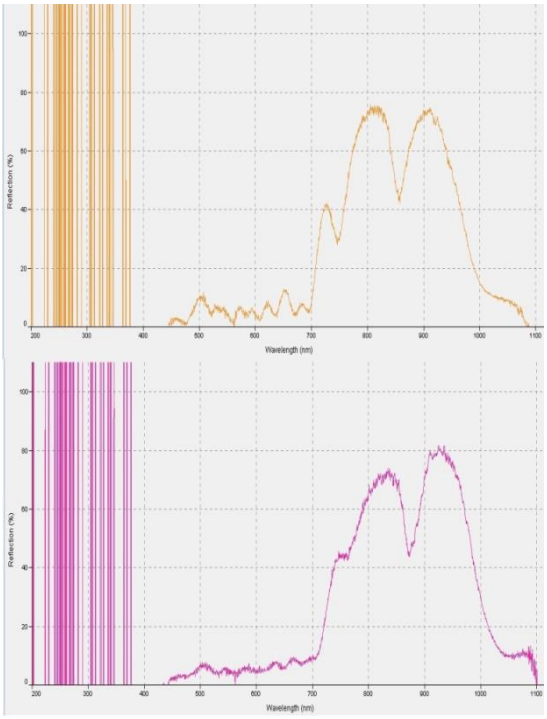

Fig. 17: Comparison of spectrum observed for biotin and 10 μm avidin

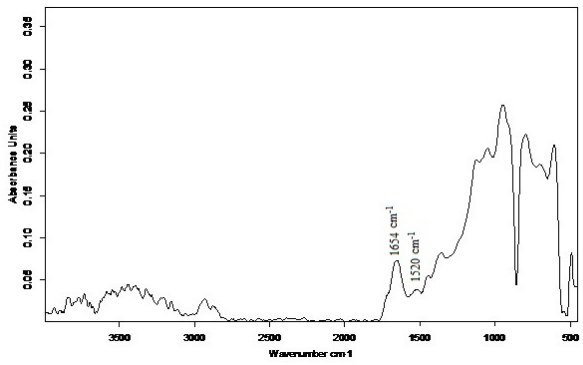

Fig. 18: FTIR Spectrum to confirm the presence of avidin protein on porous silicon microcavity structure

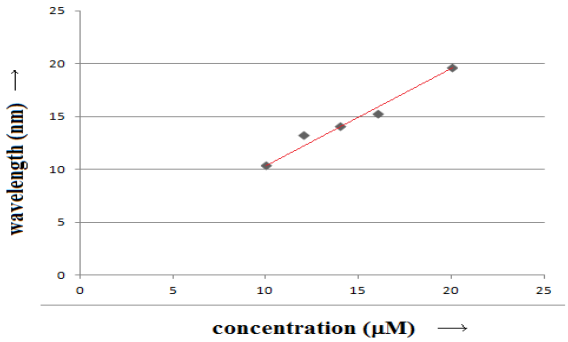

Fig. 19: Representation of wavelength shift with concentration of avidin

The two simultaneous peaks at 1654 cm^{-1} and 1520 cm^{-1} are indicative of the presence of avidin due to the amide – I bond formation during protein binding. FTIR image and spectrum obtained confirms the presence of the protein Avidin and gives credibility to the spectrum observed using the equipment (light source, spectrophotometer, Spectra Suite software), when the wavelength shift is obtained corresponding to avidin getting attached to the binding protein biotin.

A graph of wavelength shift versus concentration realized through the observations is given in Fig 19. Observing the graph tells us that wavelength shift observed for avidin concentration ranging from 10μM to 20μM is a linear one.

4. Conclusion

In this paper, a sensitive label-free optical biosensor based on PSi has been demonstrated to have the ability to detect avidin, an egg protein. PSi was fabricated to provide a base for biosensing. PS layers having different thickness and porosity were prepared, analyzed and applied to detect biomolecules. The optical properties of well-defined structures (thickness and porosity) were calculated using the principles of Distributed Bragg Reflectors and Bruggeman's Effective Medium Approximation.

The Q-factor of the microcavity, a quantity to evaluate how effectively light is confined within a microcavity structure of PSi, was obtained as 50.89.

The Scanning Electron Microscope (SEM) and Fourier Transform Infra-Red Spectroscopy (FTIR) results confirmed each stage of the fabrication of the biosensor, including the etched PSi, oxidized and functionalized using APTES, Glutaraldehyde and Biotin.

In the experiments for detection of the biotin binding protein-avidin, it was carried out for various concentrations in order to find the resolution of the fabricated biosensor. The reflectance spectrums observed were used to prove the ability of the biosensor fabricated to detect the protein. The complete detection process for a PSi base structure functionalized with the appropriate reagents takes 30-45 minutes.

For 10µM of avidin solution, a stabilized 10.4nm of shift in wavelength was observed. This was used to conclude that the resolution of the biosensor for detection of egg protein avidin was 10µM and therefore the sensitivity can be said to be 10µM.

References

[1] G. Rong, A. Najmaie, J. E. Sipe, S. M. Weiss, Biosensors and Bioelectronics, **23**, 1572 (2008).
[2] M. Khardani, M. Bouaïcha, B. Bessaïs, physica status solidi (c), **4**, 1986 (2007).
[3] Y. Koh, S. J. Kim, J. Park, C. Park, S. Cho, H. Woo, Y. C. Ko, H. Sohn, BULLETIN-KOREAN CHEMICAL SOCIETY, **28**, 2083 (2007).
[4] E. T. Vandenberg, L. Bertilsson, B. Liedberg, K. Uvdal, R. Erlandsson, H. Elwing, I. Lundström, Journal of Colloid and Interface Science, **147**, 103 (1991).
[5] E. Xifré Pérez, "Design, fabrication and characterization of porous silicon multilayer optical devices," 2007.
[6] E. A. Bayer and M. Wilchek, "11 - The avidin—Biotin system," in Immunoassay, E. P. D. K. Christopoulos, Ed., ed San Diego: Academic Press, 1996, pp. 237-267.
[7] J. Lugo, J. Faubert, M. Ocampo, and R. Doti, Porous Silicon Sensors-from Single Layers to Multilayer Structures: INTECH Open Access Publisher, 2011.
[8] O. Optics, "Spectrasuite Spectroscopy Plateform," Overture software, Inc, 2010.

*Corresponding author: vive@eced.svnit.ac.in