33rd Annual International Conference of the IEEE EMBS
Boston, Massachusetts USA, August 30 - September 3, 2011

Monolithically Integrated Mach-Zehnder Biosensors for Real-time Label-free Monitoring of Biomolecular Reactions

Eleni Makarona, Panagiota S. Petrou, Athanasia Bourkoula, Athanasios Botsialas, Maria Kitsara, Sotirios E. Kakabakos, Remco Stoffer, Gerhard Jobst, George Nounesis, Ioannis Raptis, Konstantinos Misiakos

Abstract—Arrays of monolithically integrated Mach-Zehnder interferometers were fabricated by standard silicon technology and applied to the label-free real-time monitoring of biomolecular interactions. Chips accommodating 10 MZIs were functionalized with recognition biomolecules and encapsulated in wafer scale. Detection is based on Frequency-Resolved Mach-Zehnder Interferometry, a new concept that takes advantage of the broad-band input spectrum by monitoring the changes for every input frequency. The sensitivity of the device in terms of refractive index changes (Δn) was calculated using isopropanol/water solutions. A detection limit of $\Delta n = 4\times10^{-6}$ was calculated. The bioanalytical capabilities of the device there demonstrated through model binding assays (biotin/streptavidin) as well as the detection of total prostate specific antigen in serum samples using devices coated with antigen-specific monoclonal antibody. Detection limits at the pM range were determined.

I. Introduction

THE evolution of optical biosensors to compact, small size devices appropriate for point-of-care applications has been hindered so far by the inability to incorporate all optical components onto the same substrate. Towards this direction, an optical sensor, consisting of a light emitting source, a planar waveguide and a photodetector, fully integrated on silicon substrate has been developed and evaluated as biosensor [1-3]. The transducer is fabricated by standard silicon microfabrication techniques, which allows arrays of identical transducers to be realized onto the same chip. The light emitting diode (LED) is a silicon avalanche diode that emits light over a wide spectrum in the VIS-NIR range when biased beyond its breakdown point and the photodector is a common pn-junction photodiode. These two components are optically coupled by a thin silicon nitride waveguide. Modification of the individual transducers onto the same chip by different recognition biomolecules resulted in biosensors arrays capable of multi-analyte determinations. Transduction was based on the waveguided light loss upon interaction of the immobilized recognition biomolecules with appropriately labeled biomolecules in the samples (e.g., mutation detection using fluorescently labeled PCR products [3, 4]). To move forward this sensor concept and expand its application potentials, the realization of a novel biosensor based on fully integrated-Mach Zehnder interferometers (MZIs) was considered in order to take advantage of extremely high resolution and sensitivity demonstrated by standard MZI devices [5-7]. Biomolecular reaction detection in Mach-Zehnder devices relies on the phase change occurring due to the binding of biomolecules on the surface of the sensing arm and can be in principle label-free. The devices proposed here are realized in arrays consisting of fully integrated onto Si chips MZIs, self-aligned to both the emitter and the detector, so as to avoid extensive losses in the VIS-NIR spectrum range. Each MZIs in the array has an individually operated LED, whereas all MZIs are converging to a single detector for multiplexing operation. A schematic of the integrated MZI array is depicted in Fig. 1.

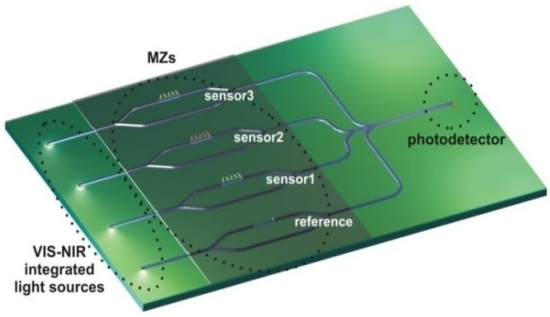

Fig. 1. Schematic of an array of monolithically integrated MZIs.

II. Operation Principle of Monolithically Integrated MZIs

A distinct difference between the proposed MZI and the pre-existing MZI sensors is that the light source employed has a broad-band spectrum covering most of the visible and near IR region [1-3] while a monochromatic light source is usually employed in the standard MZIs. Thus, in the proposed device, the monitoring of biomolecular reactions occurring on top of the sensing arm is accomplished taking

Manuscript received April 14, 2011. This work was supported by the EU Project "PYTHIA" (FP7-ICT2-224030; www.pythia-project.eu).

Eleni Makarona, Athanasios Botsialas, Maria Kitsara, Ioannis Raptis and Konstantinos Misiakos are with the Microelectronics Institute N.C.S.R "Demokritos", Aghia Paraskevi 15310, Athens Greece (corresponding author I. Raptis, phone: 00302106503226; fax: 00306515573; e-mail: raptis@imel.demokritos.gr).

Panagiota S. Petrou, Athanasia Bourkoula, and Sotirios E. Kakabakos are with the Immunoassay/Immunosensors Lab, Institute of Radioisotopes and Radiodiagnostic Products, N.C.S.R "Demokritos", Aghia Paraskevi 15310, Greece (ypetrou@rrp.demokritos.gr).

Remco Stoffer is with PhoeniX BV, Hengelosestraat, 705 7521 PA Enschede, The Netherlands (remco.stoffer@phoenixbv.com).

Gerhard Jobst is with Jobst Technologies GmbH, Engesserstrasse 4b, D-79108 Freiburg, Germany (gj@jobst-technologies.com).

George Nounesis is with BioGenomica SA, G-15232, Chalandri, Greece (www.biogenomica.gr).

into account the changes for every input frequency along the broad-band LED spectrum. Thus, a new detection concept, the Frequency-Resolved Mach-Zehnder Interferometry (FR-MZI) has been introduced in order to correlate the observed signal changes of the integrated MZI during biomolecular interactions [8, 9]. The fact that for the signal calculation the changes over the broad-band LED spectrum are used is expected to offer significant advantages over the standard single-wavelength MZIs. In particular, the drawbacks of single-wavelength MZIs that are expected to be solved by FR-MZIs are the following:

a) Optical coupling of active and passive optical components. This major issue is radically dealt with through the monolithic integration of the Si avalanche diode LED which is optically coupled to the self-aligned Si_3N_4 waveguide;

b) Suppression of signal ambiguity since the phase change for every wavelength is different and therefore it is easier to deduce from the full spectrum the exact changes;

(c) For a monochromatic source very careful design of the MZI has to be performed prior to any application in order to avoid operation near the quadrature points that can lead to signal fading. This renders each MZI applicable only to a limited number of sensing schemes. On the contrary, when a broad-band source is employed, there are wavelengths in the spectrum that will be far off from the extreme values of their corresponding transmission curves, enhancing the output signal and allowing thus the BB-MZI to be applied to a large number of detection schemes.

Moreover, the use of a broad-band spectrum is expected to provide higher detection sensitivity since every wavelength is "affected" by the binding effect in the sensing arm to a larger or lesser degree producing a phase change of its own. This in turn is translated into the output spectrum both as changes of the intensity and as peak shifts. It must be noted that the monolithically integrated LED has an emission maximum at 650 nm. Simulation experiments have revealed that the spectral region of the emission maximum is also the region for which the maximum spectral shifts are expected [10].

III. Chip Layout and Packaging

In its current format, each chip of monolithically integrated MZIs accommodates 10 sensors on an area of 9.3x4.0 mm^2 (Fig. 2). Thus, more than 150 operational chips can be received per 4-inch wafer. Chips are fabricated at the clean room facility of the Institute of Microelectronics of NCSR "Demokritos".

Each MZI in the array has its own, independently operated LED, whereas there is only one photodetector per chip, where all the MZIs converge. Special care has been taken during the design of the chip layout so that the arrangement of the 10 MZIs on the chip to minimize optical losses due to optical waveguide bending. The waveguides are covered by a 2-micron thick silicon oxide film which is removed from an area over one arm of each MZI device in the array to create the sensing arm.

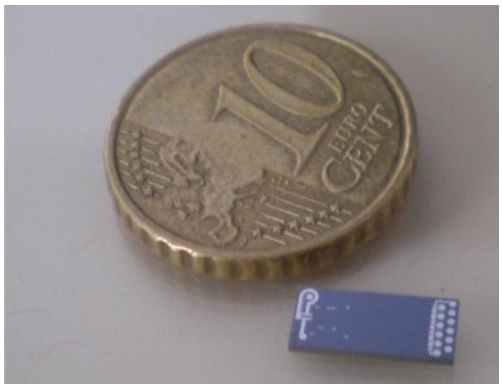

Fig. 2. Photograph of a chip accommodating 10 monolithically integrated FR-MZIs.

To convert the MZIs chip onto a biosensor array different biomolecules have to be immobilized onto the 10 MZI devices. This was accomplished by employing a microarray spotting (BioOdyssey™ Calligrapher™ miniarrayer; Bio-Rad Laboratories, Inc., USA) through appropriate programming of the instrument to achieve complete coating of the waveguides despite their non-linear arrangement. In Fig. 3, a fluorescence image obtained after spotting of the10 MZIs sensing arms on a chip with solutions of three different fluorescently labelled biomolecules is provided. To these spotting solutions 5% glycerine was added to prevent drying. However, during spotting of the active biomolecules, glycerine was omitted from the spotting solution since it leads to shrinking of the deposited solution upon drying and accumulation of glycerine which actually prevents adsorption.

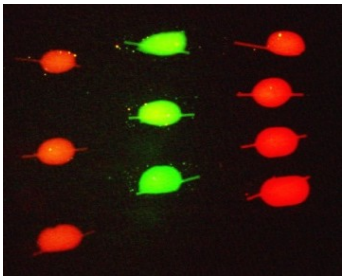

Fig. 3. Fluorescence microscope image obtained from a chip spotted with AF546 labelled anti-mouse IgG antibody (first column of three MZIs), AF488 labelled anti-rabbit IgG antibody (second column of three MZIs); and AF555 labelled streptavidin (third column of three MZIs).

The MZI chip was combined with a microfluidic module to allow for the delivery of the samples to be analyzed. The microfluidic module, shown in Fig. 4, was consisted of a polycarbonate cover bearing the fluid inlet and outlet as well as a channel for delivery of glue around the channel whereas the flow channel was photo-lithographically defined on a thin film resist (which appears blue in the photo). The fluidic was attached onto the chip first by pressing and then glued into position with a UV curable adhesive. Sealing was verified by pumping colored solutions onto the fluidic channel (Fig. 5). The assembly of the microfluidic with the

biochips could be performed at wafer scale in order to facilitate the packaging procedure thus providing a practical and good working solution to one of the bottlenecks in the development of portable bioanalytical devices based on biosensors. In addition, the implementation of mechanical machining for the preparation of the fluidic module provided flexibility with respect to materials and design during the development of the device.

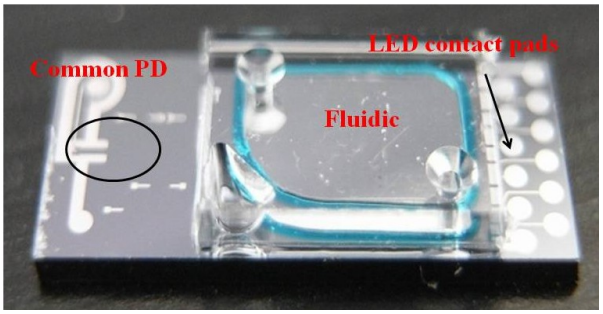

Fig. 4. Optical microscope image of an encapsulated biosensor array of multi monolithically integrated FR-MZIs.

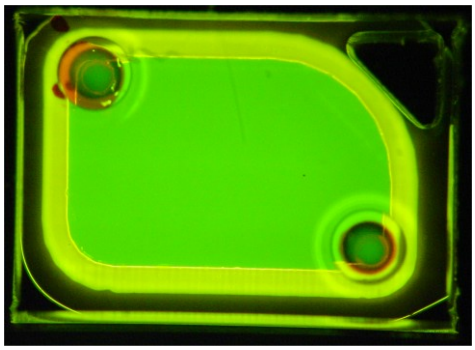

Fig. 5. Sealed fluidic on G1 chip filled with a fluorescent dye solution.

The encapsulated chips will be then fixed on a holder, Fig. 6, which will be inserted on small size instrument that will comprise the read-out electronics for signal collection and processing through appropriately designed software.

IV. Device Performance

The analytical performance of the FR-MZI devices was firstly characterized using solutions of known index of refraction. For this purpose, aqueous solutions of isopropanol were employed and their index of refraction was calculated with the aid of the Clausius Mosotti formula:

$$\frac{\eta_{sol}^{\ 2}-1}{\eta_{sol}^{\ 2}+2}=\frac{\eta_{IPA}^{\ 2}-1}{\eta_{IPA}^{\ 2}+2}(1-\phi)+\frac{\eta_{water}^{\ 2}-1}{\eta_{water}^{\ 2}+2}\phi$$

where n_{sol}, n_{IPA} and n_{water} are the indices of refraction of the solution, isopropanol and water and **φ** is the concentration of the water with respect to the isopropanol content. The values of n_{IPA}=1.378 and n_{water}=1.333 for the red part of the VIS spectrum were used in the calculations. Since the accuracy of the electrometer used is 0.01 pA, the minimum current change that can be safely detected was set to 3 times this value. According to the experimental data the smallest detectable change of ΔI = 0.04 pA corresponded to a change of the refractive index of $\Delta n = 4\times10^{-6}$ (or ~$\Delta N \sim 5\times10^{-7}$). The sensitivity of the fully integrated chips is linear, and from fits the slope was evaluated to 5500 pA/RIU. Therefore, the LOD (Limit of Detection) of the fully integrated chips can be set to $\Delta n = 4\times10^{-6}$ with a resolution $\delta n \sim 5.45\times10^{-6}$. The apparent discrepancy of ΔI and slight deviation from the linear behavior of the chips for the highest isopropanol concentration used (which corresponded to $\Delta n = 4\times10^{-4}$) is due to the fact that for such a high change of the refractive index, the photocurrent after almost a full sinusoid was very close to a local extremum (minimum) where the derivative is close to zero. This detection limit put the proposed FR-MZI interferometric sensor amongst the most sensitive sensors based on refractive index changes monitoring.

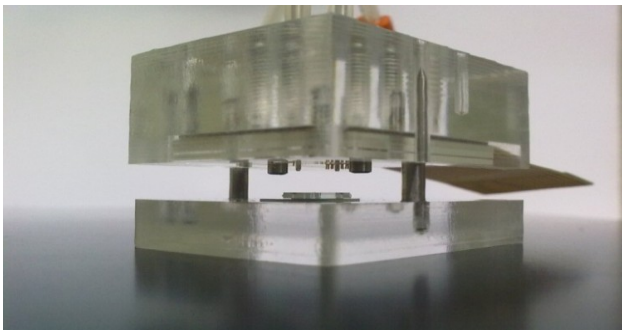

Fig. 6. The encapsulated biochip placed in the probing head.

V. Label-free Detection with Monolithically Integrated MZIs

The bioanalytical capabilities of the developed sensor were further evaluated through a model binding assay based on biotin/streptavidin interaction. For this purpose, chips were coated with a 0.4 μM biotinylated bovine serum albumin solution in 50 mM phosphate buffer, pH 7.4, and then the surface free protein binding sites were blocked using a 150 μM bovine serum albumin solution in 50 mM phosphate buffer, pH 7.4. After that, the chips were tested using streptavidin solutions with concentrations ranging from nM to pM range all diluted in phosphate buffer, pH 7.4, containing 150 μM bovine serum albumin. The binding of streptavidin to immobilized biotin moieties was monitored in real-time and in label-free format.

As it is shown in Fig. 7, there is a distinguished response for streptavidin concentrations as low as 10 pM. The signal slope during reaction was proportional to the streptavidin concentration used. Thus, there is no need to rely on maximum plateau values, which in case of very low concentration might require substantial time intervals to achieve, in order to correlate the signal with the analyte concentration, but use instead the signal change at the first minutes of reaction. Thus, the developed biosensor can be used for extremely rapid detection of biomolecular reactions

after appropriate modification of the integrated MZI sensing arm with recognition biomolecules.

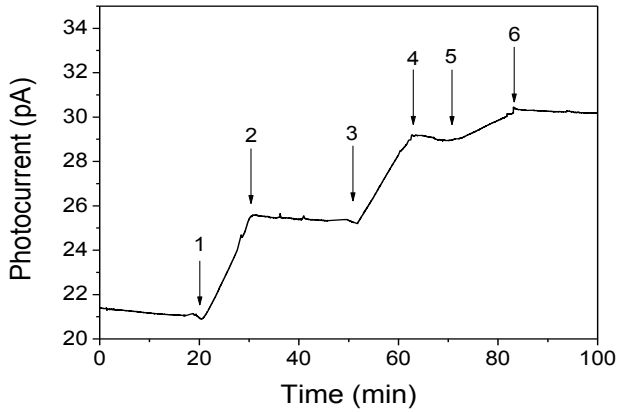

Fig. 7. Real time response of an MZI sensor coated with biotinylated BSA upon running: start to arrow 1: 50 mM phosphate buffer, pH 7.4; arrow 1 to 2: 50 pM streptavidin solution; arrow 2 to 3: 50 mM phosphate buffer, pH 7.4; arrow 3 to 4: 25 pM streptavidin solution; arrow 4 to 5: 50 mM phosphate buffer, pH 7.4; arrow 5 to 6: 10 nM streptavidin solution; arrow 6 to end: 50 mM phosphate buffer, pH 7.4. The flow rate was 20 μl/min.

The developed sensor array chips is currently used for the simultaneous and highly sensitive detection of total- and free-prostate specific antigen (PSA) in human serum. For this purpose, distinct sensors of the same array will be modified with specific mouse monoclonal antibodies against total- or free-PSA (Fig. 8). The aim is to develop a sensor that can be used not only for the simultaneous detection of prostate cancer based on total- and free-PSA detection in human serum samples but also for follow-up of prostatectomised patients. Preliminary results of total and free-PSA determinations showed that the detection limits of the FR-MZI chips are in the order of a few ng/ml and comparable to other state-of-the-art label-free biosensors, such as microring resonators [11], albeit with the added value of the monolithic integration of all optical components on a single disposable chip.

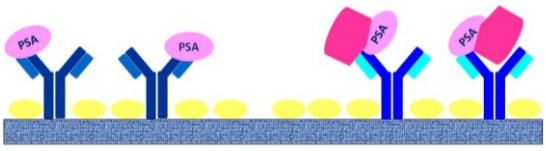

Fig. 8. Schematic of simultaneous detection of total- and free-PSA in human serum using FR-MZI chip arrays functionalized with analyte specific capture antibodies.

VI. Conclusion

A fully integrated onto silicon substrate Frequency-Resolved Mach-Zehnder Interferometric sensor was presented. Efficient and practical approaches for chip functionalization with biomolecules and fluidic interface were also developed. The sensor was calibrated with respect to refractive index changes that can be detected using aqueous isopropanol solutions. A limit of detection equal to $\Delta n = 4\times10^{-6}$ was determined. The reaction of streptavidin with immobilized onto the sensing arms biotin moieties was monitored in real time and label-free format at concentrations as low as a few pM. The application of the developed sensor for the simultaneous and sensitive detection of multiple analytes in the same sample can provide an elegant solution towards the development of compact, portable and cost-efficient biosensors and point-of-care devices.

Acknowledgment

This work was supported by the EU Project "PYTHIA" (FP7-ICT2-224030; www.pythia-project.eu). Authors would like to thank Mr. Emmanuel Sergis and Christina Georgiou for their valuable help in the fabrication of the devices.

References

[1] K. Misiakos, S. E. Kakabakos, P. S. Petrou, and H. H. Ruf, "A Monolithic Silicon Optoelectronic Transducer as a Real-Time Affinity Biosensor," Anal. Chem. vol. 76, pp. 1366-1373, 2004.

[2] K. Misiakos, E. Mavrogiannopoulou, P. Petrou, and S. Kakabakos, "Monolithic Silicon Optical Microdevices for Biomolecular Sensing," presented at the IEEE Sensors 2009 Conference, Christchurch, New Zealand, October 25-28, Conference Proceedings pp. 17-20.

[3] K. Misiakos, P. S. Petrou, S. E. Kakabakos, D. Yannoukakos, H. Contopanagos, T. Knoll, T. Velten, M. DeFazio, L. Schiavo, M. Passamano, D. Stamou, and G. Nounesis, "Fully integrated monolithic optoelectronic transducer for real-time protein and DNA detection: The NEMOSLAB approach," Biosensor Bioelectron. Vol. 26, pp. 1528–1535, 2010.

[4] E. Mavrogiannopoulou, P. S. Petrou, S. E. Kakabakos, and K. Misiakos, "Real-time detection of BRCA1 gene mutations using a monolithic silicon optocoupler array," Biosensor Bioelectron. Vol. 24, 1341–1347, 2009.

[5] R.G. Heideman, P.V. Lambeck "Remote opto-chemical sensing with extreme sensitivity: design, fabrication and performance of a pigtailed integrated optical phase-modulated Mach–Zehnder interferometer system," Sensor. Actuator. B vol. 61, pp. 100-115, 1999.

[6] A. Densmore, M. Vachon, D.-X. Xu, S. Janz, R. Ma, Y.-H. Li, G. Lopinski, A. Delâge, J. Lapointe, C. C. Luebbert, Q. Y. Liu, P. Cheben, and J. H. Schmid, "Silicon photonic wire biosensor array for multiplexed real-time and label-free molecular detection," Optics Lett. vol. 34, pp. 3598-3600, 2009.

[7] F. Prieto, B. Sepïlveda, A. Calle, A. Llobera, C. Domvnguez, A. Abad, A. Montoya, L.M. Lechuga "An integrated optical interferometric nanodevice based on silicon technology for biosensor applications," Nanotechnology vol. 14, pp. 907-912, 2003.

[8] I. Raptis, K. Misiakos, S. Kakabakos, P. Petrou, E. Makarona, M. Kitsara, "Monolithically Integrated Physical Chemical and Biological Sensor Arrays based on Broad-band Mach-Zehnder Interferometry," PCT, WO 2009/115847 A1, September 24, 2009.

[9] M. Kitsara, K. Misiakos, I. Raptis, and E. Makarona, "Integrated optical frequency-resolved Mach-Zehnder interferometers for label-free affinity sensing," Opt. Ex. vol. 18, pp. 8193-8206, 2010.

[10] P. S. Petrou, M. Kitsara, E. Makarona, I. Raptis, S. E. Kakabakos, R. Stoffer, G. Jobst, and K. Misiakos, "Monolithically Integrated Biosensors based on Frequency-Resolved Mach-Zehnder Interferometers for Multi-analyte determinations," presented at the 2010 Annual International Conference of the IEEE Engineering in Medicine and Biology Society, EMBC'10 , Buenos Aires, Argentina, August 31- September 4, Conference Proceedings pp. 298-301.

[11] A. L. Washburn, M. S. Luchansky, A. L. Bowman, and R. C. Bailey, "Quantitative, Label-Free Detection of Five Protein Biomarkers Using Multiplexed Arrays of Silicon Photonic Microring Resonators", Anal. Chem., vol. 82, pp. 69-72, 2010.