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Integrated optical biosensor for rapid detection of bacteria

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Abstract: In medical diagnostics, rapid detection of pathogenic bacteria from body fluids is one of the basic issues. Most state-of-the-art methods require optical labeling, increasing the complexity, duration and cost of the analysis. Therefore, there is a strong need for developing selective sensory devices based on label-free techniques, in order to increase the speed, and reduce the cost of detection. In a recent paper, we have shown that an integrated optical Mach-Zehnder interferometer, a highly sensitive all-optical device made of a cheap photopolymer, can be used as a powerful lab-on-a-chip tool for speci?c, label-free detection of proteins. By proper modifications of this technique, our interferometric biosensor was combined with a microfluidic system allowing the rapid and specific detection of bacteria from solutions, having the surface of the sensor functionalized by bacterium-specific antibodies. The experiments proved that the biosensor was able to detect *Escherichia coli* bacteria at concentrations of 10^6 cfu/ml within a few minutes, that makes our device an appropriate tool for fast, label-free detection of bacteria from body fluids such as urine or sputum. On the other hand, possible applications of the device may not be restricted to medical microbiology, since bacterial identification is an important task in microbial forensics, criminal investigations, bio-terrorism threats and in environmental studies, as well.

Keywords: Biosensor, Integrated optics, Mach-Zehnder interferometer, microfluidics, *E. coli*, bacteria

1 Introduction

Bacteria analysis and the identification of microorganisms to the species level and beyond is a key task of microbiology. Accurate and definitive microorganism identification, including bacterial identification and pathogen detection, is essential for the correct disease diagnosis, treatment of infection and trace-back of disease outbreaks associated with microbial infections. Traditionally this is achieved by carrying out labour-intensive and time-consuming biochemical assays. Traditional methods of bacterial identification rely on phenotypic identification of the causative organism using stainings (for example: Gram-staining), culture and numerous biochemical methods and antibiotic susceptibility test. Many of these are used decades ago, although in recent years there has been a need on updating or revising the already existing methodology. The traditional microbiologic methods of microbial detection tend to be labor-intensive and take more than a day to obtain results and even more to get the antibiotic susceptibility results. These have resulted in numerous rapid techniques. Rapid methods for microbial detection can be sensitive, precise, and relatively quick. However, these modern methods are more expensive than their conventional counterpart, and the direct cost of the test or in the time to prepare the sample for testing takes more. Although it is well-known by the users that obtaining results more quickly is worth the cost of these systems.

Rapid microbiological tests

Rapid microbiological tests and reagents range from simple biochemical or immunological techniques to direct specimen tests to a combination of sophisticated instrumentation, software, consumables, and reagents (as recent reviews, see [1, 2]). Specific detection, quantitative, or identification methodologies may be employed, depending upon the desired approach, epidemiological significance, and degree of identification required. Many of the current rapid tests and reagents on the market today re-

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quire a relatively large number of living microbial cells be present in the sample. Moreover, since testing may be performed in a variety of locations— hospital settings, or research and reference labs—it is important to understand the technical capabilities that are required. Those include the method sensitivity and specificity, expectations of the system or method, and the degree of training required of the operator.

One type of common rapid test method involves the use of miniaturized biochemical identification devices. These disposable devices perform a range of biochemical tests simultaneously. They are generally designed to identify specific bacterial species. These units simplify conventional testing procedures by eliminating the need for multiple tubed or plated media; they are generally space, time, and cost savers.

Some rapid tests and reagents involve using antibodies or nucleic acids to detect pathogens or toxins. Of these, antibodies are the most versatile and may be used in a variety of kits. These rapid kits take advantage of antibody-antigen interactions unique to a particular microorganism. A latex agglutination test works in this manner: beads are coated with plasma antibodies unique to a particular pathogen. If the reaction turns positive, the latex beads will cause the bacterium to clump or agglutinate. If the beads are colored and are placed on a white background, it is easy to detect a positive reaction by observing visible clumping.

Enzyme-linked immunosorbent assays and enzyme immunoassays rely on antibody-antigen interactions which can be placed on a microplate or combined on a membrane and incorporated into a lateral flow kit. Antibody screening and toxin tests are examples of these types of kits and are most useful for direct specimen testing when a result is needed quickly to aid in the diagnosis of disease.

Polymerase chain reaction-based kits (PCR) use an enzyme to replicate a small portion of a target microorganism's DNA. The reaction involves attaching a marker to the target DNA so that it is more easily visualized. The advantage of this rapid test format is that very small numbers of a pathogen can be detected rather quickly. However, PCR-based methods cannot distinguish between viable and nonviable cells, so results must be interpreted accordingly.

These are just a few examples of what rapid test and reagent kits can do, but the goal of rapid testing is the same no matter the application: to provide accurate identification and rapid communication to interested parties in order to improve outcomes. Some test kits can provide results within four to 24 hours.

Cost may be the main downside to some of the newest forms of rapid tests: there is a price to pay for research and development and to promote innovative technologies. For larger laboratories, economics of scale and reduced labor costs may offset the cost per test: speedy results carry a cost benefit. Conversely, costs may be significant enough to put some of the more recent high-tech rapid methods out of reach for smaller laboratories, especially when there is no foreseeable cost benefit in results or if the reading window does not fit into a smaller laboratory's scheduling restrictions. This is also in conflict with recent requirements, when real-life measurement systems, like POCT (Point Of Care Testing), NPT (Near Patient Testing) are emerging in more and more areas of clinical pathology, infectivology, toxicology, food and environmental protection. Robustness of the analytical techniques should meet the requirement of having operating personnel with only low or medium level of training in the area of biomedical analytics. Hence, there is an urgent need for cost-effective detection methods allowing automated data collection and processing.

In this paper, we report on the development of a lab-on-a-chip tool for rapid detection of bacteria from small amounts of fluids. The device is built around a biosensor adapted to this purpose, based on an integrated optical Mach-Zehnder interferometer [3]. After giving a short introduction to its operation priciple, we give a detailed description of the structure of the device, and show the results of test measurements demonstrating the functionality of the tool. Potential applications of the results in medical diagnostics and beyond, is also discussed.

2 Materials and Methods

2.1 Principle of operation

Integrated optical interferometric biosensors combine the detection of evanescent field with the measurement of phase alteration.

The operation is based on the specific binding of the target molecule or bacteria to the measuring arm of the interferometer. This binding is realized by appropriate recognition elements – for example receptors, antibodies, enzymes - immobilized to the waveguide surface. The binding of target objects causes a local refractive index change at the sensor surface which effects the propagation of the incoupled light in the waveguide via the evanescent field. The resulting phase difference between the propagating mode in the measuring and reference arms can be de-

tected as an output intensity change due to the interference (Fig. 1).

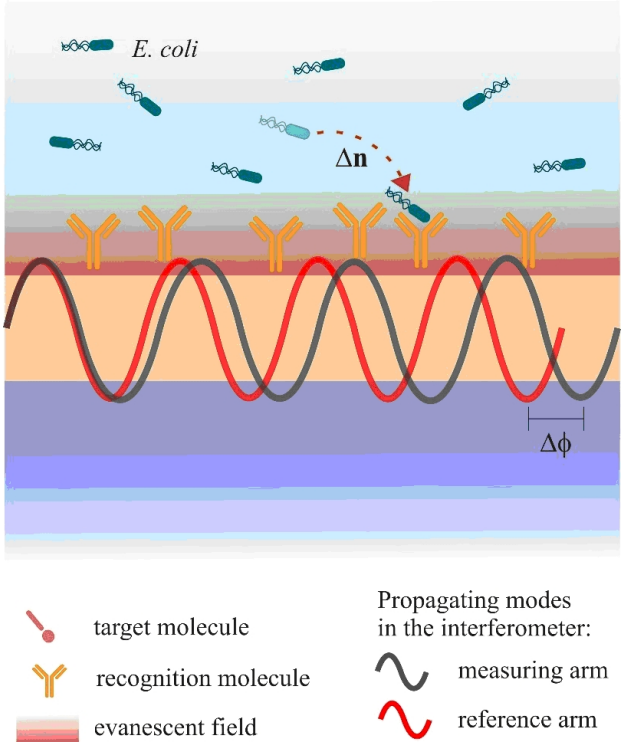

Figure 1: The operation of an interferometric biosensor is based on the specific binding of the target molecule or bacteria to the measuring arm of the interferometer. When the target object binds to the to the surface a local refractive index change takes place, resulting phase difference between the propagating mode in the measuring and reference arm. The resulting intensity change at the output can be detected.

The transmission function of the interferometer is sinusoidal, as it was demonstrated in [3] by illuminating an auxiliary thin (4 μm) film of the chromoprotein bacteriorhodopsin (bR) layered onto the reference arm of the interferometer (see also in Fig. 2, below). Using saturating light intensities (e.g., 100 mW/cm^2 at 532 nm), two full periods (4π phase shift) could be covered, that makes the bR film suitable for adjusting the operating point of the interferometer to the highest sensitivity (i.e., to the mean of the minimum and maximum output values) [3]. As we have shown it earlier [4], under such conditions, the refractive index change of the bacteriorhodopsin film is ca. $5{\cdot}10^{-3}$. When using the integrated optical interferometer for biosensing purposes, however, by at least an order of of magnitude smaller changes in the effective refractive index of the adlayer are estimated to occur upon the initial binding of a sparse layer of proteins or cells on the surface of the waveguide structure [5]. This implies that, during the sensing process, the response of the interferometer remains in the linear range. In addition, as pointed out in [6], in first approximation the effective refractive index change of the adlayer is proportional to the change of the adsorbed mass, and to bulk concentration of the analyte. Hence, the initial slope of the signal detected by our interferometric sensor should be proportional to the concentration of the bacteria to be detected.

2.2 Preparation of the biosensor

2.2.1 Integrated optical MZI

The Mach-Zehnder interferometer (MZI) was prepared by photopolymerization of NOA81 optical adhesive (Norland Products Inc., USA) by a so called direct laser writing technique that we used already for the preparation of micromachines and integrated optical structures [4, 7]. Briefly, a microscope coverslip was spincoated by the adhesive (2100 rpm, 120 s), and subsequently mounted on the motorized X-Y stage (Märzhauser) of an inverted microscope (Zeiss Axiovert 200) for writing. The beam of a laser diode (NDV4313, Nichia Co., 405 nm, 20 mW) was focussed by a microscope objective (10X, 0.25NA) onto the NOA81 layer. By properly moving the motorized stage, the interferometer structure was exposed into the layer. Where necessary, the writing was stopped by blocking the laser beam by a shutter (Uniblitz VS14S2ZM1R1-21, Vincent Associates). Both the motorized stage and the shutter were controlled by a computer. The cover slip was then thoroughly rinsed in a 1:3 mixture of Ethanol and Acetone, in order to remove the unexposed parts. The width of the line of the interferometer (2 μm) was controlled by adjusting the focal spot size of the microscope objective and the writing speed. In order to have proper input and output facets, the ends of the waveguide were cleaved by the help of a diamond cutter (S90W, Thorlabs Inc.). Finally, the coverslip was glued on a microscope slide also by NOA81 exposed by a 100-W mercury arch lamp. The optical connection was established by single-mode optical fibres (S630-HP, Thorlabs). A beam of a laser diode (633 nm, 10 mW) was then coupled into the optical fiber, whose other end was matched to the input of the MZ interferometer by a micropositioner (DC-3K, Märzhäuser Wetzlar GmbH & Co. KG, Germany), and its optimal position was fixed by a photopolymer glue (OP-66-LS, Dymax Europe GmbH, Germany). Outcoupling from the MZI was accomplished the same way (Fig. 2).

For measuring the transfer characteristics of the interferometer the red light of a laserdiode (633 nm, 10 mW)

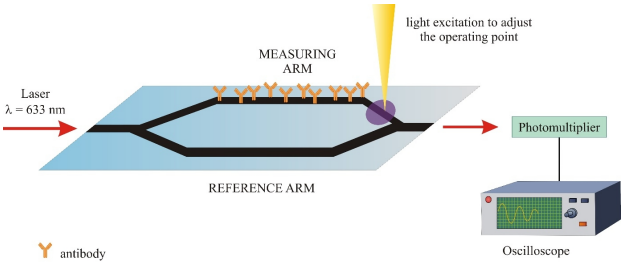

Figure 2: Schematic picture of the setup. Red light of a laserdiode (633 nm, 10 mW) was coupled to the interferometer, the transmitted light was guided to a photomultiplier and the signal was recorded by a storage oscilloscope.

was coupled to the interferometer through the attached single mode optical fibre. The output optical fibre guided the transmitted red light to a photomultiplier (H5783-01, Hamamatsu, Japan) and the signal was recorded by a storage oscilloscope (WaveRunner 6100A, LeCroy, USA).

2.2.2 Fabrication of PDMS microfluidic channels

For the test experiment with the immunological sensor, microfluidic channels were prepared of PDMS by soft lithography. First the mold was made from SU8 photopolimer using negative photolitoghraphy. During the process a spincoated, soft baked (90 °C, 5 min) SU8 layer (30 µm) was exposed by UV light ($\lambda \approx$ 365 nm, P ≈ 76 mW/cm^2, Newport, Oriel 97435 UV lamp) through a photomask. Then liquid PDMS prepolymer (degassed 1:10 mixture of Sylgard 184, Dow Corning) was poured over the master and cured for 30 miutes at 80°C. The PDMS replica was then peeled from the master and holes were punched for pipette tips at inlets and outlets. Thereafter the PDMS was sealed above the Mach-Zehnder interferometer.

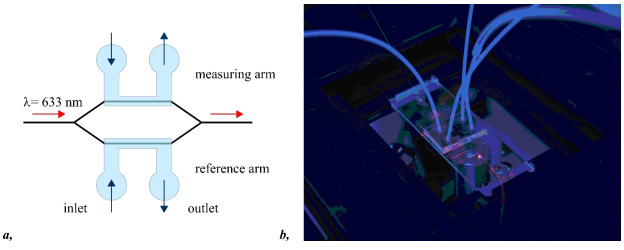

Figure 3: a, Schematic illustration of the microfluidic channel above the Mach-zehnder interferometer. The inlet and outlet holes were formed far from the waveguide to avoid possible damages. b, Photography of the ready made biosensor (silicone tubing and optical fibers are attached)

To form the seal, the PDMS and the interferometer were exposed to oxygen plasma (PDC-002, Harrick Plasma), where 400 mtorr=53.3 Pa oxygene pressure was maintained and 29.6W RF power was applied for 60 seconds. Then the PDMS block was carefully positioned and attached above the parallel parts of the Mach-Zehnder. To improve the strength of the seal, the device was kept overnight in an incubator at 40 °C. Finally pipette tips were fitted into the inlet and outlet holes and silicone tubing (d = 1 mm) was attached (Fig. 3).

2.2.3 Waveguide functionalization

The measuring arm was functionalized to achieve the specific binding of the target bacteria *Escherihica coli* DH5 alpha. For filling up the microfluidic channels syringe pump and silicone pumping were used.

Before starting the functionalization the PDMS channels were cleaned with ethanol. Then the measuring arm was filled with 2% APTES solution ((3-Aminopropyl)triethoxysilane 99% Sigma-Aldrich, diluted with isopropanol) for 90 mins. After this the channels were leached with isopropanol for 10 mins to remove unbound APTES. The channels were dried with air and washed with MilliQ water to remove physiabsorb APTES. The device was cured at 100 °C for 6 mins on a hot plate to strengthen the binding between APTES and the waveguide surface. The second step was the filling of the channels with 2.5% gluteraldehyde solution (Grade II, 25% in H_2O, Sigma-Aldrich, diluted with PBS (1X, pH 7.4)) for 2hours. Thereafter the device was washed thoroughly with PBS for 10 mins. Then the channels were filled with the antibody solution (Anti-*E. coli* antibody - Azide free (Cat. No.: ab48416), Abcam; diluted with PBS) for overnight. Next day the device was leached with PBS to remove the unbound antibodies. As a result of the functionalization procedure an antibody adlayer formed on the surface of the measuring arm.

2.2.4 *E. Coli* culture

Colonies of *E. coli* Dh5-alpha strain were maintained on agar plates at 4°C. Before the experiments, one colony was placed into 3 ml LB (lysogeny broth) medium in a sterile polystyrene tube, and the bacteria were grown overnight in a shaker incubator at 30°C. Overnight cultures were diluted back in the morning 100 times, and cells were grown until they reached an optical density of 0.5–0.8 at 600 nm (OD 600). 1 ml of the cell culture was centrifuged (3000

rpm, 10 min) and resuspended in PBS (phosphate saline buffer) solution.

2.2.5 Testing the binding ability of anti-*E. coli* antibody with ELISA

The binding ability of the goat polyclonal anti-*E. coli* IgG antibody (Abcam, Cat. No.: ab48416) was tested with an in house constructed indirect ELISA. For the test, *E. coli* DH5α strain was propagated on an agar plate for 24 hours. The colonies were collected with an inoculating loop and a suspension was made in phosphate buffered saline (PBS). The ELISA plate (R&D Systems, Cat. No.: DY994) was coated overnight at 4°C with the serial dilution of the *E. coli* suspension in a range from 10^1 to 10^6 fold dilution in a presence and absence of 0.5% formaldehyde (Reanal, 10492-1-01-65). After coating, the fluid was aspirated and the plate was blocked with 1% bovine serum albumin (Sigma, Cat. No.: A-6793) for two hours at room temperature followed by washing it three times with 0.05% Tween20 (Sigma, Cat. No.: 93773) in PBS. The anti-*E.coli* antibody was added as a primary antibody in 1 µg/ml and 2 µg/ml concentrations and incubated for one hour at room temperature. The plate was washed three times with 0.05% Tween20 in PBS. The bounded primary antibody was detected with a peroxidase labelled rabbit anti-goat IgG antibody (Sigma-Aldrich, Cat. No.: A5420) in 40000 fold dilution. After one hour incubation at room temperature the plate was washed five times with 0.05% Tween20 in PBS followed by adding the TMB substrate (Abcam, Cat. No.: ab171522). After 25 minutes the reaction was stopped with 2N sulphuric acid (R&D Systems, Cat. No.: DY994) and the plate was measured at 450 nm with the reference filter of 690 nm. Formaldehyde helped the immobilization of *E. coli* antigens on the plate surface during the coating.

3 Results and discussion

3.1 ELISA measurements

Prior to the biosensing experiments, the binding ability of the goat polyclonal anti-*E. coli* IgG antibody was tested with an in-house constructed indirect ELISA.

The experiments proved that the tested anti-*E. coli* antibody could bind the *E. coli* antigens. The amount of the bounded primary antibody depended on its concentration, the coating buffer and the amount of antigens on the plate. The optical density was higher by use of higher concentrations of anti-*E. coli* antibody, as well as by larger amounts of the antigen.

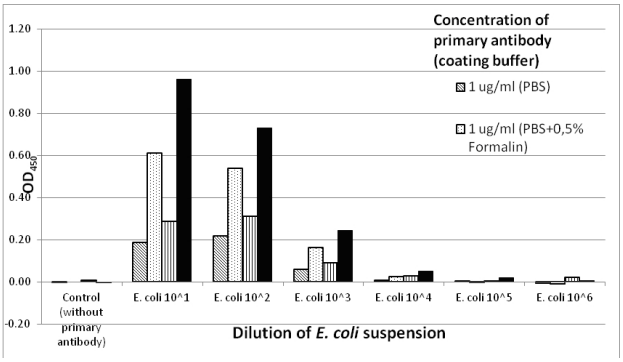

Figure 4: Testing the binding ability of the anti-*E. coli* antibody with ELISA with different concentrations of primary antibody and coating buffers (OD: optical density)

3.2 Interferometric detection

The measurements were started after the above functionalization process. Prior to the experiments, the operating point of the device was set appropriately to achieve the highest sensitivity (see 2.2.1). Then, the *E. coli* suspension of $4{\cdot}10^{-6}$ cfu/ml concentration was pumped into the measuring reference channels at time zero. Fig. 5 shows subsequent intensity changes of the light outcoupled from the MZI. The time course of the light intensity change was fitted by a single exponential, whose time constant was ca. 600 s, which was attributed to the binding process of bacteria to

the measuring arm of the interferometer. The arrangement of the setup allowed a continuous visual control of the binding process by a microscope. Fig. 6 shows a section of the measuring arm with bacteria bound to the surface. At the same time, no binding was observed at the reference arm of the MZI (data not shown).

Note, however, that visual observation by the microscope was used only to control that binding process goes parallelly with the development of the ourput signal, it is not an essential part of our device, whose arrangement supports a full automatic control of the sensing process, from the sample delivery to the detection of the signal. The integrated optical structure combined with microfluidics, on the other hand, grants portability of the device, fulfilling the requirements of state-of-the-art POC diagnostics [8].

The time constant observed for binding is in a good agreement with data of other studies got for characteristic times of cell adsorption to solid surfaces [9, 10]. Note, how-

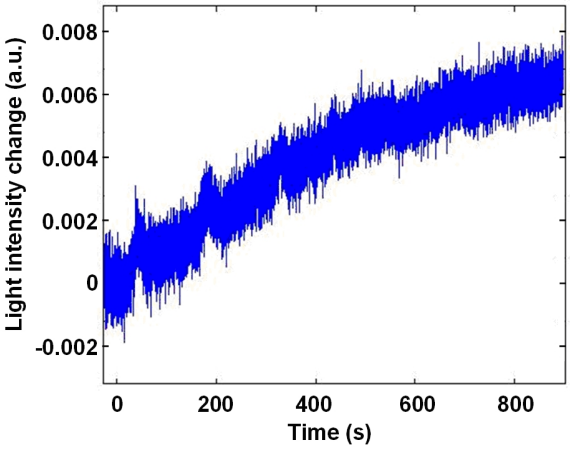

Figure 5: Light intensity change at the output of the Mach-Zehnder interferometer after pumping *E. coli* bacterium suspension above the measuring arm at time zero. The zero value of the Y axis refers to the mean of the minimum and maximum light intensities of the sinusoidal transmission function of the interferometer, where the operating point was adjusted prior to the measurement (see also 2.1). The curve could be fitted with a sigle exponential with a time constant of 600 s (not shown).

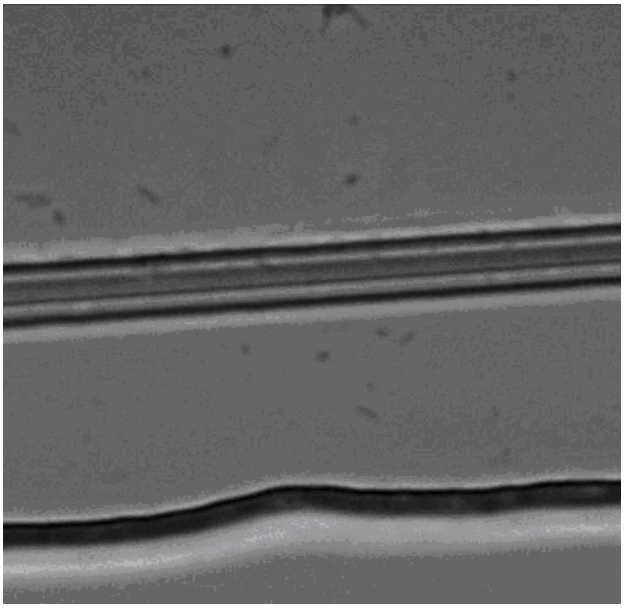

Figure 6: Photograph of a section of the biosensor after binding of the coli bacteria (dark spots) to the functionalized surface. The upper stripe is part of the measuring arm of the MZI, while the lower one is showing the microchannel wall.

ever, that the binding rate of the analyte should be proportional to its concentration (see 2.1). Although the exact calibration of our biosensor is to be the subject of follow-up studies, according to our estimate based on the present experiments, the detection limit in bacterium concentration is ca. 10^6 cfu/ml, which is comparable to characteristic pathogene concentrations in sputum and urine [11, 12], implying that our device is appropriate for screening such body fluids. However, bacterium concentration in septic blood is usually not exceeding 10^3 cfu/ml, meaning that further improvement of the sensitivity of our technique is necessary for testing blood samples. Note, at the same time, that bacterial identification is used not only in medical microbiology, but also in microbial forensics, in criminal investigations, in bio-terrorism threats and in environmental studies as well.

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