Doctoral School of Multidisciplinary Medical Science
Albert Szent-Györgyi Medical School
University of Szeged

# Developing integrated optical structures, with special respect to applications in medical diagnostics

## Summary of the Ph.D. Thesis

**Dániel Petrovszki**

Supervisors:
András Dér PhD, DSc; scientific advisor
Sándor Valkai PhD; research associate

Biomolecular Electronics Research Group
Institute of Biophysics
Biological Research Centre

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# List of publications

## List of papers directly related to the subject of the thesis

I. **Petrovszki D,** Valkai S, Gora E, Tanner M, Bányai A, Fürjes P, & Dér A. An integrated electro-optical biosensor system for rapid, low-cost detection of bacteria. Microelectronic Engineering. 2021; 239,111523. IF: 2.662

II. **Petrovszki D***, Krekic S*, Valkai, S, Heiner Z, & Dér A. All-Optical Switching Demonstrated with Photoactive Yellow Protein Films. Biosensors. 2021; 11(11),432. IF: 5.743

III. **Petrovszki D***, Walter FR*, Vigh JP, Kocsis A, Valkai S, Deli MA, & Dér A. Penetration of the SARS-CoV-2 Spike Protein across the Blood–Brain Barrier, as Revealed by a Combination of a Human Cell Culture Model System and Optical Biosensing. Biomedicines. 2022; 10(1), 188. IF: 4.757

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# 1. Introduction

Recently, biosensors have become an essential part of the medical diagnostics toolbox. In this context, pregnancy tests or blood glucose meters have been in everyday use. The purpose of the biosensors is to detect analytes from biological samples or body fluids (e.g., saliva, blood, urine) rapidly, specifically, sensitively, and reliably, based on a change in a physical signal (electrical, optical, mass, etc.). Such biological target particles can be proteins, viruses, bacteria, etc. For some biosensors, labelling is also used for detection (e.g., fluorescent dyes) to achieve high sensitivity. However, their label-free versions have sufficient sensitivity and ensure cost-effective operation, which make them promising candidates for various applications. For example, they have the potential to be used as rapid tests in medical diagnostics, providing a suitable alternative to traditional laboratory tests. Thus, a given pathogen or pathological condition can be detected rapidly in a user-friendly, portable way. This can be of high importance for rapid diagnosis and appropriate treatment at the point-of-care (POC). Moreover, this favorable feature can also be used for ‘on-site’ testing, whereby the diagnostic test can be performed in households. The importance of such applications has also been demonstrated during the recent pandemic period (COVID-19) caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), where rapid tests played a key role both in controlling the spread of the disease and in rapid diagnosis, which could sometimes be lifesaving. To implement the above-mentioned features in a portable, easy-to-use and highly sensitive single chip platform, biosensors can be incorporated in lab-on-a-chip (LOC) devices for label-free and real-time ‘on-chip’ analysis. In this way, small amounts of biological fluid samples can be handled, delivered to the sensor, and then the pathogens in that sample can be rapidly, specifically, and sensitively detected on the same chip.

In terms of the practical use of the biosensors for such POC purposes, label-free optical ones offer a promising alternative. Due to the design of their integrated optical (IO) constructions, these types of biosensors enable the detection of pathogens in the desired miniaturized, portable, and sensitive manner. In such systems, light propagates in a waveguide structure by total internal reflections, which provides the basis of the detection through changes in its properties. During propagation, light also penetrates to thin layers of the surrounding media, which is called the evanescent field. Analytes binding to the biosensor surface in this field result in a change in the phase of the light propagating in the waveguide, or in its scattering into the surrounding medium. These interactions can be exploited for the specific detection, by pre-covering the

surface of the waveguide with bio-recognition elements, thus performing its so-called functionalization. As a result, not only rapid and sensitive, but even specific detection of the analyte of interest is feasible, as utilized in various ways in the majority of integrated optical biosensors. Among them, interferometric designs are widely used, one of the most sensitive ones being the so-called Mach-Zehnder interferometer (MZI) setups. In these, light travels in a single inlet waveguide section, which then splits, guiding the light in two arms of equal length - reference and measuring arms -, which are subsequently joining in a single outlet. Binding of pathogens to the surface of the measuring arm causes a phase change of the light propagating through it, whose superposition with the reference beam causes interference, resulting a change in output light intensity. Incidental disturbing effects due to changes in environmental parameters (e.g., temperature or humidity) during the course of measurements, can be compensated by tuning the operating point (bias) of the interferometer. An effective way to achieve this is done via a thermo-optical control of the refractive index in the reference-arm environment.

One of the key parameters of biosensors is their sensitivity, which is influenced by the concentration of analytes in the sensing region. Accordingly, integrated optical biosensors can reach higher sensitivity by increasing the amount of pathogens in the evanescent field at the surface of the waveguide. Taking advantage of the polarizability of the target cells, this can be achieved by using the phenomenon of dielectrophoresis (DEP). This technique is widely used in impedance-measurement based biosensors, for collecting larger-size analytes (e.g., extracellular vesicles or cells) on the surface electrodes. In my experiments, I also applied this technique integrated in a biosensor system based on evanescent sensing.

# Objectives

My PhD work was focused on the design and fabrication of integrated optical structures with the aim of their potential use in medical diagnostics. In this context, integrated optical biosensors were developed and applied for pathogen detection from fluid samples via evanescent field sensing. These can provide the basis of POC, cost-effective clinical rapid tests. For their realization, the devices were designed to integrate waveguides into a microfluidic channel on a glass substrate ([T1, T2]).

1. As for the first application, an optical biosensing detection approach was chosen. The aim was to create a device, which was capable of label-free, rapid, selective, and sensitive bacteria detection from body fluids. The detection concept was based on the scattering of evanescent waves of red light by target cells. Analysis of the resulted scattering pattern allows the determination of the bacteria concentration in the fluid sample.
    - I wanted to demonstrate the usability of the measurement principle with quantitative detection of *Escherichia coli (E. coli)* bacterial cells in suspension.
    - The sensitivity of such a device can be increased by using dielectrophoresis. It exploits the polarizability of the cells, collecting them onto the surface of electrodes in the vicinity of the waveguide, applying alternating electric field.
    - The size dependence of the dielectrophoretic force on particles allows selective collection and detection of pathogens. To test this, I aimed to detect bacteria in artificial urine sample containing somatic cells, mimicking inflammatory conditions.
2. As a second application, I aimed to develop a miniature Mach-Zehnder interferometer-based biosensor for the specific, quantitative detection of viral proteins. Due to the interferometric measurement principle, rapid, accurate detection of the proteins can be achieved. I intended to use the device to investigate the potential effects of the neuroinvasion of coronavirus (SARS-CoV-2) infection. The crossing of biological barriers by the virus and its subunits is a current and intensively researched subject. Recently, SARS-CoV-2 has been found to transcellularly cross mouse and hamster primary brain endothelial cell culture models, without altering the expression of tight junctions. It was hypothesized that the S1 subunit in mice crosses the blood-brain barrier (BBB) by adsorption-mediated transcytosis. Our biologist colleagues have previously developed several *in vitro* systems to model the human BBB and other biological barrier systems. Since COVID-19 may also be associated with gastrointestinal symptoms, we wanted to extend the experimental setup to the gut epithelium, as well, for which Caco-2 cell culture provided the appropriate cell model.
    - Specially, an integrated optical biosensor was planned to be developed, capable of determining the ability of coronavirus surface spike protein S1 subunit to penetrate *in vitro* models of human blood-brain barrier and intestinal epithelial barrier. Therefore, experiments were designed to use the biosensor for specific, quantitative

detection of spike proteins that may have crossed the barrier models in permeability assays.

To achieve specific detection, the aim was to functionalize the waveguide surface of the interferometer with a specific S1 protein antibody (MonoRab™, BS-R2B2, GenScript).

- To reach optimal, stable detection, the bias-point adjustment of the sinusoidal transfer function of the interferometer can be used. To this end, I wanted to thermo-optically modulate the refractive index of the medium in the proximity of the waveguide. This can be achieved by applying a direct current (DC) to a gold microheater structure placed near the reference arm of the interferometer.

# 2. Materials and methods

## 2.1. Design and fabrication of biosensors

Both biosensor construction, presented in my PhD thesis, were built from the same components. Gold surface microstructures and polymeric waveguides were realized on a microscope coverslip, combined with a silicon-based polymeric microfluidic channel. The design and fabrication of the devices followed the same process, with minor variations depending on their application. Firstly, the surface gold microstructures and then polymer waveguides were created on the surface of the glass substrate. The patterns of the structures were realized in layers of ultraviolet light sensitive epoxy-based polymers, so-called photopolymers, or photoresists, spincoated onto the substrate, using a maskless direct laser-writing photolithography technique (µPG-101 instrument, λ = 375 nm, Heidelberg Instruments GmbH). Both positive and negative photoresists were used for structuring, following the manufacturer's protocols. (In the former, cross-links are broken by illumination in the layer, in the latter, cross-links are formed by the same effect.) First, masks were formed with patterns of surface gold microstructures in positive photoresist (in the electro-optical biosensor, this was an electrode array of electrode pairs, while in the integrated optical interferometer, it was a heating wire structure placed to the reference arm of the interferometer). Then, sputtering technique was used to deposit a homogeneous layer of chromium and then gold on the glass substrate in the pattern, determined by the mask. The waveguide structures were then made on this glass substrate containing the gold microstructures. For red light, a waveguide strip capable of propagating multiple light modes in the gap between electrode pairs in the electro-optical

biosensor, and a single-mode Mach-Zehnder interferometer pattern in the second application, were created using the same direct laser writing technique. In both cases, the structures were prepared from photopolymers developed for waveguide fabrication, in the first case from EpoCore and in the second case from SU-8 negative photopolymer materials (micro resist technology GmbH). Then, the coverslip, containing the biosensor components, was covered with a silicon-based poly-dimethyl-siloxane (PDMS) microfluidic channel, so that the waveguide structures underlying the detection were placed in the channels. The microfluidic channels were prepared by soft-lithographic technique, and then were bonded to the glass substrate after oxygen plasma treatment. During the measurements, the syringes containing the fluid sample were connected with pipette tips and rubber tubes inserted into the inlet and outlet ports of the channels.

## 2.2. Waveguide biofunctionalization for specific detection

The specific recognition of the S1 subunit of the spike protein by the IO MZI biosensor was achieved by the immunological antigen-antibody interaction on the waveguide surface of the measuring arm, coated with protein-specific antibodies. For this purpose, the channels were filled with antibody-activating reagent (Mix&Go solution diluted 1:20 with MilliQ deionized water, AnteoTech Ltd.) once they were washed with ethanol. Next, they were incubated for 30 min at room temperature. Then, the MZI arms were treated first with phosphate-buffer saline (PBS) (1x, pH=7.4), then with SARS-CoV-2 specific antibody solution (MonoRab™, BS-R2B2, GenScript, diluted with 1×PBS at a final concentration of 5 μg/ml). The samples were incubated overnight at 4 °C. Prior to measurements, both the washing of the unbound antibodies and the local refractive index matching for both MZI arms were performed. For this, the buffer (0.1% bovine serum albumin (BSA)-Ringer-HEPES (RH) buffer), derived from the permeability assays, was used.

## 2.3. Preparation of fluid samples containing bacteria

The detection concept, optimization and performance of the electro-optical biosensor were tested with cultured suspensions containing different concentrations of live *E. coli* bacterial cells (non-virulent Dh5α strain). For this purpose, bacteria were cultured on agar plates, then the suspensions of these cells were prepared in lysogeny broth (LB) at different cell concentration dilutions ($10^2$ -$10^6$ colony forming units (CFU) per milliliter). The concentration

of bacteria in the undiluted bacterial culture fluid was determined (CFU/ml values) by plate counting technique. The master dilution solution of the suspensions used for the measurements contained LB and deionized MilliQ water in a ratio of 1:9. This provided the conditions for DEP-based cell collecting, meanwhile maintained the viability of the cells in the sample.

An artificial urine sample containing a mixture of living somatic endothelial (hCMEC/D3) and bacterial cells was prepared and used to test the selectivity of the DEP-based cell collection technique and the practical application of the electro-optical biosensor. For this pilot test, the LB was replaced by an artificial negative urine sample in the master dilution solution.

## 2.4. Experimental setups for the optical biosensors

For the measurements with the integrated optical biosensors, the particular device was placed on a motorized stage equipped with a micropositioner (DC-3K, Märzhäuser) of an inverted microscope (Zeiss Axiovert 200). A CCD camera, attached to the microscope, was used to monitor the processes and to capture images for bacterial detection. To flow the applied fluids in the microfluidic channels, the sample in a syringe was injected by a pump (SP210IWZ syringe pump, World Precision Instruments Inc.) through silicone tubes to the inlets via pipette tips attached to them. For both sensors, electrical wires were connected to the outlet contact pads of the surface gold structures (microheater or electrode system). For the electro-optical biosensor, the DEP-based cell collection was achieved by applying sinusoidal alternating (AC) electric field of different frequencies to the electrodes, utilizing the signal of a function generator (20 MHz model 8020, Tabor Electronics). For triggering a square wave signal (1.4 s 'ON' and 3.0 s 'OFF') from a timer (Uniblitz VS14S2ZM1R1-21, Vincent Associates) was used. The bias point of the interferometer-based biosensor was adjusted by applying 0-4.6 V of a DC power supply (VLP 2403pro, Conrad Electronic), using a thermo-optical method, by heat, generated on the surface gold microheater. The red light from the laser diode was coupled to the input of the corresponding integrated optical biosensor with a single-mode optical fiber (S630-HP, Thorlabs) using an end-coupling technique. Similarly for the MZI sensor, the light at the output of the interferometer was transmitted to a photoelectron multiplier (H5783-01, Hamamatsu). Its signal was then delivered to and recorded by an oscilloscope (LeCroy 9310-L, LeCroy). In each case, the measurements with each biosensor were repeated at least twice.

# 3. Results and discussion

## 3.1. Electro-optical biosensor for bacteria detection [T1]

### 3.1.1. Optimization of dielectrophoretic particle collection

To increase the sensitivity of bacterium detection by the electro-optical biosensor, DEP-based cell collection was performed. Thus, this technique was optimized prior to detection, with a construction consisting of thin-film surface-electrode pairs placed in the microfluidic channel. In this term, DEP-based particle collection capability and its size-dependent selectivity were studied. First, a mixture of microbeads of various diameters (1 µm, 9 µm), suspended in deionized water, was flowed through the channel, while an AC electric field (30 V peak-to-peak, 5 MHz) was applied on the electrodes. Under such conditions, the smaller beads were collected at the surface of the electrodes, in the gap between the electrode-pairs, while the bigger ones flowed through the channel without being trapped. In the next step, the target particles were *E. coli* bacteria in suspensions. Considering the differences between the dielectric properties of bacterial cells and microbeads, as well as the ionic strengths of the solutions, I varied the applied electric field parameters and the dilutions of the bacterial suspension solvent to investigate the performance of the cell collection electrode system.

The alternating field frequency was varied between 100 kHz and 5 MHz, and the dilution of the solvent was varied between 1x and 100x. Bacteria were collected for 30 min at a bacterial concentration of $10^4$ CFU/ml. Based on microscopic observations, the default frequency of 5 MHz, and 10x dilution of ionic strength proved to be ideal, so I used these parameters in subsequent studies (30 $V_{p\text{-}p}$, 5 MHz, sinusoidal signal).

### 3.1.2. Evaluation of measurements

The main goal was to perform the evaluation of the target-cell detection measurements without a high magnification microscope, thus without the limitation of observing the individual target cells for efficient measurements. Considering this aspect, I detected the dielectrophoretic capturing of the cells from their suspension, by monitoring the scattering pattern of evanescent waves upon adsorption of cells on the optical waveguide formed between the electrodes, using an objective of 20x magnification. Meanwhile, the electric field was periodically switched off (‘OFF state’) and then on (‘ON state’) for short periods, to modify the adhesion between the waveguide surface and the target cells. This resulted a change in the scattering pattern of the evanescent waves, which was recorded by the CCD camera of the

microscope setup. This measurement concept provided the basis for quantitative detection of bacterial cells from images captured in different states of the system (OFF and ON). The resulting image pairs were statistically processed, focusing on the quantitative description of the differences between ON and OFF patterns of scattered light using the MATLAB software. For this purpose, the mean square error (MSE) and the correlation values were calculated for each image pair. With this technique, the total detection time was about 40 minutes.

Further tests were carried out to determine the resolution at which the difference in the pattern of the recorded scattered light images could still be considered significant. Therefore, images were captured and analyzed with different objectives (x4.7, x10, x20 magnification) at a bacterial suspension of $10^4$ CFU/ml concentration. The results showed that there was a significant difference between the MSE values of the control, and the measurement image pairs, reflecting bacteria movement, even for images taken with lower magnification objectives (x4.7, x10). Based on these findings, it was concluded that this detection approach can be applied with a low-cost web camera, which also envisage a portable construction of this biosensor.

#### 3.1.3. Estimation of the detection limit

Given the optimized measurement parameters (x20 objective, 30 $V_{p\text{-}p}$, 5 MHz, sinusoidal AC electric field), tests were performed to estimate the sensitivity of the biosensor device. To this end, a concentration series of *E. coli* cells ($10^2$ -$10^6$ CFU/ml) was used, and then the patterns of the light scattered on cells collected on the waveguide surface were detected. I found that, compared to the MSE values of the control images, the values of the measurement image pairs ("OFF" and "ON" state pairs of captured images) were significantly different even after 10 min of cell collection, and cell concentration as low as $10^2$ CFU/ml, considerably reducing the overall measurement time. However, this difference was not found significant for the sample of 10 CFU/ml; hence the concentration of $10^2$ CFU/ml was estimated as the detection limit. The difference in MSE values could also be used for sensor calibration, as a suitable calibration curve ($R^2$ = 0.91) was possible to fit to the resulting data.

#### 3.1.4. Tests with artificial urine containing somatic cells

The practical applicability of the DEP-based bacterium cell collection method was also tested in a medium modeling inflammatory urine, infected with bacteria, and containing other somatic cell types. Therefore, the focus of this pilot study was on the selectivity of the cell collection procedure. For this investigation, artificial urine containing a mixture of *E. coli* ($10^4$

CFU/ml) and hCMEC/D3 endothelial cell suspensions was flowed through the channel, while an AC electric field (30 $V_{p-p}$, 5 MHz) was applied. It was observed that this field had distinct effects on different cell sizes, due to the size and frequency dependence of the dielectrophoresis. Bacteria (~1-2 μm) were collected at the electrode surfaces, on the waveguides, while endothelial cells (~10 μm) flowed through the channel without being trapped. In conclusion, the selectivity of this cell collection technique was demonstrated under physiologically relevant conditions.

## 3.2. Integrated optical Mach-Zehnder interferometer for the detection of coronavirus S1 subunit passage through biological barrier systems [T2]

### 3.2.1. Thermo-optical bias point adjustment

During the thermo-optical bias point adjustment of the Mach-Zehnder interferometer, the DC voltage applied to the microheater was steadily increased from 0 V to 4.6 V, while the light intensity of the interferometer output was measured (red and green light, 633 nm and 532 nm, respectively). With the applied heating voltage, the output signal at 532 nm, and later at 670 nm, wavelength was determined. In this technique, the heating causes a change in the local temperature of the waveguide environment, which leads to a modulation of the output light intensity by shifting the phase of the light propagating in the reference arm of the MZI. By varying the voltage, the light intensity can thus be adjusted to one of the inflection points of the transfer function, where the detection sensitivity is highest. In this way, the baseline drift, caused by slow changes in environmental conditions (e.g., temperature and humidity), can be compensated for the duration of the measurement (a few minutes).

### 3.2.2. Measurement results

First, calibration sample solutions containing the S1 subunit of the spike protein at two different concentrations (2 and 20 μg/ml in 0.1% BSA-RH buffer) were flowed through the channels of the measuring arms. Meanwhile the change in light intensity due to protein adsorption was detected through a single-mode optical fiber attached to the optical output of the interferometer. Subsequently, similar measurements were performed with fluid samples from permeability assays of the target protein, carried out in case of two biological barrier system models. These fluid samples derived from the lower compartment of the assay, potentially containing the proteins that were able to cross the barriers from the upper compartment, where a 200 μg/ml stock solution was injected. In addition, experiments were

performed with control samples. For example, the negative control was obtained from models without spike proteins, and the positive control was a sample containing spike proteins that passed from the S1 stock-solution in the upper part of the cell-free models to the lower part, through the separating porous membrane.

As it was expected, the negative control sample resulted a near-zero background signal, as it did not cause a significant change in output light intensity of the sensor. In contrast, the positive control showed high peak amplitudes, which were found to be almost identical to the signal of the more concentrated (20 μg/ml) calibration sample. Target solutions from the two barrier models showed much lower, and different, estimated protein concentrations. The signal of the biosensor in case of the BBB model was approximately equal to that of the 2 μg/ml calibration solution. Unalike, the signal level was lower for the case of Caco-2 cell model system. Based on the signals of the calibration samples, the concentration value for the latter could be estimated to be about 0.5 μg/ml. The observed, reduced permeability of the S1 spike protein subunit through the Caco-2 cell model is likely to be due to different passage pathways in epithelial cells.

These results were also confirmed by ELISA tests on the same samples, indicating that the label-free, specific, sensitive detection of spike protein passage through barrier systems was achieved in a rapid measurement time of only a few minutes. Furthermore, this IO MZI biosensor can be prepared in a portable and cost-effective manner, which also facilitates its applicability as a POC testing tool for early, effective detection of the virus, or related proteins.

## 4. Summary

Accomplishing the first objective of my PhD-work, an integrated electro-optical biosensor was designed, fabricated and applied for image-based, label-free, rapid bacteria detection. Its working principle was demonstrated with live, non-virulent *E. coli* bacterial suspensions. The sensing concept was based on bacteria detection via the evanescent field, in the vicinity of a waveguide structures. The sensitivity of this technique was enhanced by the application of dielectrophoresis, thus collecting the target cells on the waveguide surface, prior to detection. The parameters of the alternating electric field used for cell collection and the sample solutions were optimized. Image processing of the scattering patterns recorded with the on/off states of the electric field enabled quantitative detection. The detection method does not require such resolution that is necessary to observe individual cells, since the differences in scattering patterns are providing suitable basis for efficient sensing. I have shown that reliable image

processing results can be obtained with the use of objective of similar magnification (x4.7) to a low-cost web camera, supporting the cost-effective implementation of this device. The performance of the presented detection concept was shown to be promising, reaching a detection limit of $10^2$ CFU/ml, which is a relevant value for the characteristic concentrations of some pathogens in body fluids (e.g. urine). In addition, with the measurement time of 10 minutes (including cell collection), the method is considerably shorter than the time needed for performing conventional bacteria screening laboratory techniques requiring a cultivation step. Although the biosensor was primarily used for non-specific detection of bacteria, the feasibility of size-selective detection by dielectrophoretic cell collection was proved in several cases. By functionalizing the waveguide surface of the biosensor, specific detection can also be achieved, and the image analysis procedure provides further possibilities for improvement.

Based on the demonstration of this proof-of-concept detection method, this biosensor design has some promising aspects regarding its sensitivity, low cost of fabrication, and rapid detection process. These could all contribute to its application as a point-of-care diagnostic test in the future.

As the second objective of my studies, an integrated optical Mach-Zehnder interferometer-based biosensor was realized to be applied in a study focusing on the evaluation of the biological barrier-penetration capability of the SARS-CoV-2 surface spike protein S1 subunit. To model this process, *in vitro* biological barrier systems of two human organs highly exposed to coronavirus infection, the brain and the intestine, were selected. Thus, the experiments were focused on the passage of the spike protein fragment across blood-brain barrier models and intestinal epithelium cell-culture systems. Biosensing measurements were performed with S1 protein-containing fluids derived from permeability assays, where the protein penetration capability across the barrier system models was tested. Thus, the sensor was used to achieve sensitive, specific, and rapid quantitative detection of the amount of protein passing through the barrier systems. To maintain the optimal operation and sensitivity of the interferometer, thermo-optical bias point adjustment was applied. The measurements with the biosensor provided accurate determination of the amount of S1 protein that passed through the barrier systems. Furthermore, it was established that the S1 protein could pass through the two types of barriers in different amounts. The results of the experiments with the biosensor were in agreement with the results of conventional immunological tests (ELISA) carried out in parallel. Thus, the application of the integrated optical Mach-Zehnder interferometer biosensor demonstrates that this detection approach can be used for similar medical diagnostic purposes and can contribute to the investigation of the adverse effects of SARS-CoV-2 on the human body.