

**Project closing report
(final report)**

**Optical resonances in functionalized protein assemblies
for label-free sensing**

by

Robert Horvath

Project details:

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Panel: FIZ

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Summary of results in English

After 7 years of intensive research in Denmark and in England the present fellowship gave Robert Horvath the possibility to come back to Hungary and join the Research Institute of Technical Physics and Materials Science (MTA MFA). At the host institute a long term strategic plan is to build up a strong research direction in the field of biological and chemical sensing and conduct basic and applied research in these fields. During the present project not only novel label-free biosensor configurations with extremely high sensitivities were realized, but using the developed and commercially available biosensor platforms (OWLS) the self-assembly and surface behavior of biological thin films and living cells were investigated too. As an interesting finding, oriented protein adsorption was revealed by monitoring the birefringence of monomolecular flagellin films. The same methodology was applied to measure exchange processes in supported lipid bilayers without using any molecular labels. The self-assembly of protein-polyelectrolyte films were also followed at the nanometer scale, proving - for example - that the protein flagellin can be easily incorporated into oppositely charged polymeric films; potentially creating functional nanolayers with receptor or enzymatic properties.

Summary of results in Hungarian

A jelen OTKA projekt lehetőséget nyújtott arra, hogy Horváth Róbert 7 évig tartó, Dániában és Angliában folytatott kutatómunkája után hazatérjen. Az MTA MFA fogadó intézményben hosszú távú stratégiai cél a biológiai és kémiai szenzorok alap illetve alkalmazott kutatásának a megerősítése. Az elvégzett kutatómunka eredményesen illeszkedett ezekhez az elképzelésekhez. A projekt keretében nem csupán nagy érzékenységű jelölésmentes optikai bioszenzorok sikeres fejlesztése zajlott, hanem felhasználásukkal és kereskedelmi szenzorok (OWLS) segítségével biológiai filmek és élő sejtek önszerveződése, felületi viselkedése is tanulmányozásra kerültek. Egy fontos eredményként bizonyítást nyert, hogy a felületre adszorbeálódó fehérje filmek optikai kettőtörésének vizsgálatával a molekulák felületi orientációjáról hasznos információ nyerhető. Ugyanennek a metodikának a segítségével kicserélődési folyamatok tanulmányozhatóak lipid kettősrétegekben, mindenféle jelölőmolekula alkalmazása nélkül. Továbbá, fehérje-polielektrolit kompozit filmek önszerveződésének vizsgálata során kimutatásra került, hogy a flagellin fehérje sikeresen beépíthető ellentétesen töltött polimer filmekbe. Ezen eredmények egy új lehetőséget nyújtanak flagellin alapú funkcionális bevonatok előállítására, amelyek például receptor vagy enzimatikus tulajdonságokkal rendelkeznek.

Summary of major research findings

Development of biosensor instrumentation

An interferometric sensor based on gratings on a planar optical waveguide is introduced. The device combines the advantages of known interference-based waveguide sensors with the simplicity of grating couplers. In the presented configuration, two parallel and coherent light beams, laterally separated in the direction of mode propagation, are coupled into a planar waveguide through a grating. One of the coupled beams is phase modulated using a periodically relaxing liquid crystal modulator, resulting in a time varying intensity signal at the end face of the waveguide. Refractive index changes within the waveguide section between the two coupling regions are monitored by observing characteristic changes in the intensity signal. We started to test the developed instrument in biosensing experiments. This work is still ongoing, but already resulted in accepted publications in *Applied Physics B* and in *Sensors and Actuators B*. We have successfully created multichannel devices too, when one of the channels can be used for internal referencing. This development significantly extends the practical applicability of these sensors. One publication is under preparation at the moment and planned to be submitted to *Optics Express* or to a similar high quality optics journal. We have also started to use the developed device for on-line monitoring bacterial adhesion on various surfaces.

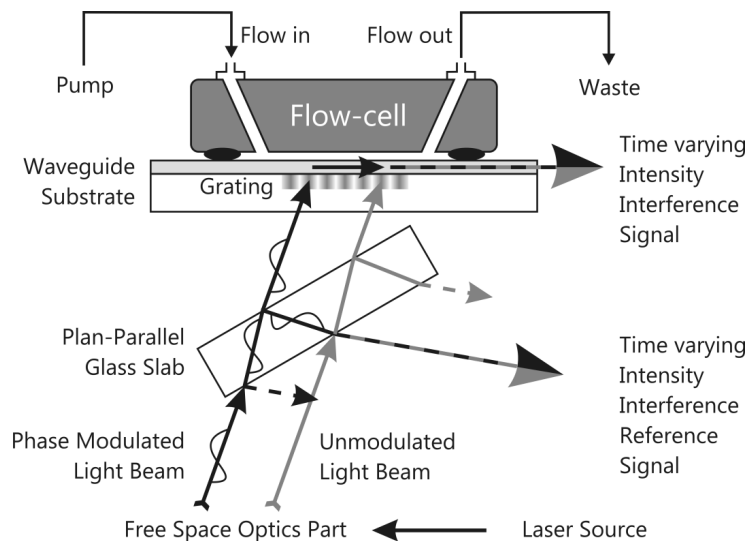


Figure 1.

Cross section of the developed biosensor device.

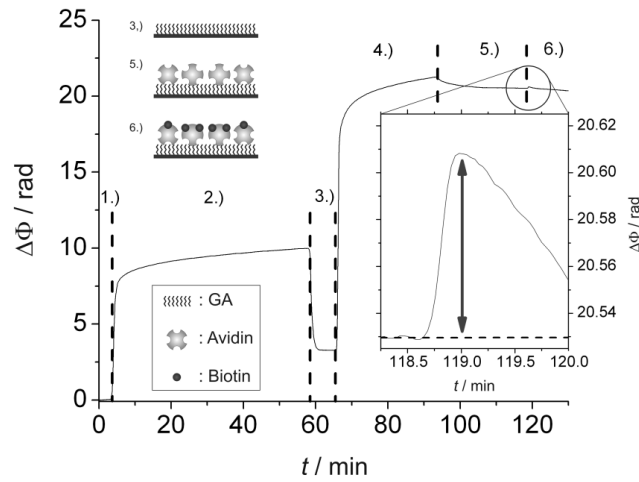


Figure 2.

Demonstration of biotin binding to an immobilized avidin layer. The experiment demonstrates the extremely high sensitivity of the sensor. Note, the detection of the low molecular weight biotin (244 Da) is usually impossible with classical label-free biosensor devices.

Flagellin adsorption monitored by optical waveguides

The surface adsorption of the protein flagellin was followed in-situ using Optical Waveguide Lightmode Spectroscopy (OWLS).

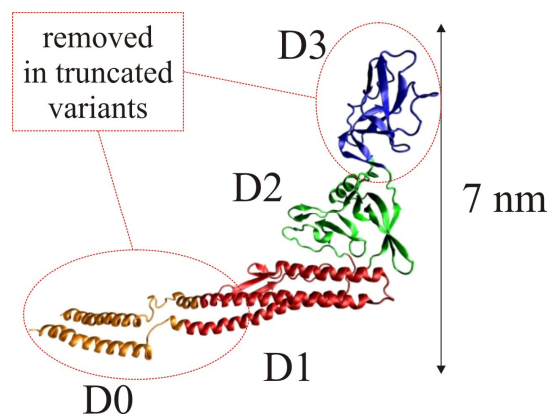


Figure 3.

The structure of the flagellin in the flagellar filament. The protein contains four domains (D0, D1, D2 and D3). In solution the terminal domains D0 and D1 are disordered, but the other parts of flagellin are compact well folded domains. The removed parts of the truncated flagellin variants are also indicated. The whole D0 and some parts of the D1 domain are removed in case of F40, while the central D3 domain is removed in $\Delta D3_FliC$.

Flagellin did not show significant adsorption on the hydrophilic bare waveguide, but very rapidly formed a dense monolayer on the silanized hydrophobic surface. The homogeneous and isotropic optical layer model - generally applied for adsorbed protein films - failed to characterize the flagellin layers, but could be successfully modeled as uniaxial thin films. The introduced anisotropic modeling revealed a significant positive birefringence in the layer, suggesting oriented protein adsorption. The adsorbed flagellin orientation was further evidenced by monitoring the surface adsorption of truncated flagellin variants (see **Fig. 3.**), in which the terminal helical regions or the central D3 protein domain was removed. Without the terminal helixes the protein adsorption slowed down and the resulting films were significantly less birefringent (**Fig. 4.**), implying that flagellin adsorbs on the hydrophobic surface through its terminal helixes (see **Fig. 5.**).

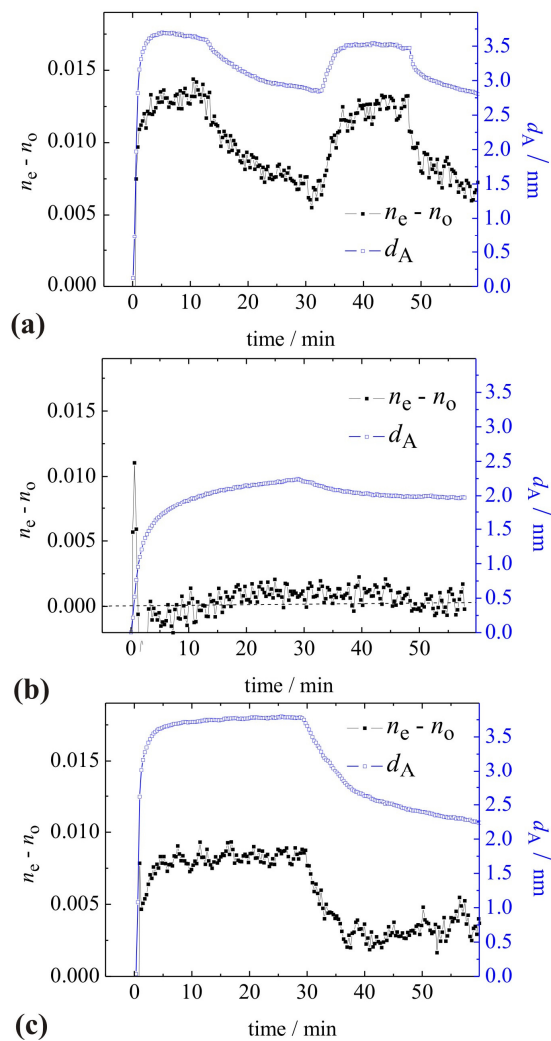


Figure 4.

Birefringence and averaged thickness of the adsorbed layers treating them as uniaxially anisotropic. (a) flagellin, (b) F40 and (c) $\Delta D3_FliC$

Until now parts of these results were published at the *Nanobio Zurich 2010* conference as a poster presentation and a student research work (TDK in Hungarian) was submitted to the Technical University of Budapest. One peer-reviewed publication is under preparation, which is planned to be submitted to *Analytical Chemistry* or to *Langmuir*.

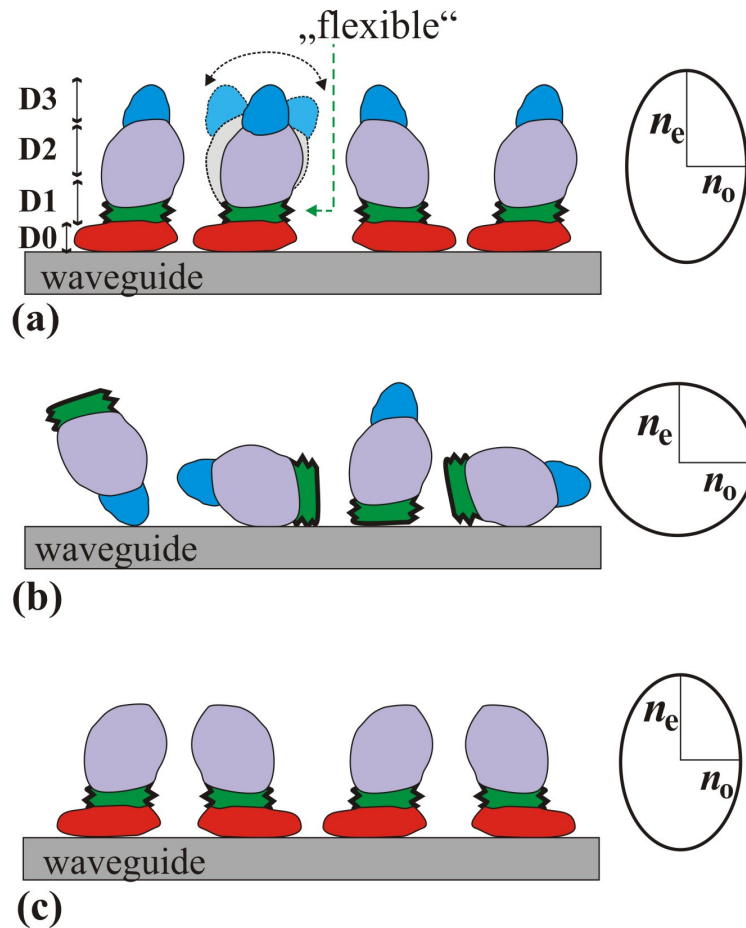


Figure 5.

Schematic illustration of the internal structure of the adsorbed protein layers. (a) flagellin (b) F40 and (c) $\Delta D3_FliC$. The refractive index ellipsoids are also shown (at right). The terminal part of the D1 domain is disordered in solution (indicated by an arrow in (a)); upon surface adsorption of the proteins this part forms a flexible joint between the D0 domain and the other well folded and structurally compact parts of the proteins.

The above developed methodology was used to investigate exchange processes in supported lipid bilayers in close collaboration with Prof. Éva Kiss (Eötvös University). Two publications are under preparation in this topic.

OWLS biosensor measurements using low sample volume

The OWLS (Optical Waveguide Lightmode Spectroscopy) technique gives the possibility to monitor cellular or molecular processes (such as cell adhesion, receptor-ligand interactions, adsorption of proteins etc.) in real time without the need of additional labeling. Setups working on the OWLS principle are nowadays considered to be one of the most sensitive biosensors, and therefore the technique is widespread having significant scientific interest.

The fluidic setup connected to the OWLS optical unit is usually built up from several different elements; syringe pump, tubes, connections and sealing units in between the tubes, bubble traps, junctions, and flow-through cuvette can be present. The type, number, and arrangement of these fluidic components depend on the type of measurement planned to be made. Due to its complexity, the fluidic setup is a critical part of the whole OWLS system, and beyond that, it is a potential source of errors and artifacts when the samples or flow rates are changed.

Even more complications arise when the available amount of sample is strongly limited, for example, when expensive bioreceptors, samples from living organisms, cell cultures are measured. Supervising a master student from the Eötvös University (Norbert Orgován) Robert Horvath studied the possibilities to reduce the sample volume in OWLS measurements. For this reason the flashing of the OWLS cuvette was investigated systematically using various tubing length, liquid injection systems and model solutions. A student research work (TDK in Hungarian) was submitted dealing with this topic and the publication as a technical note in a sensor journal is foreseen.

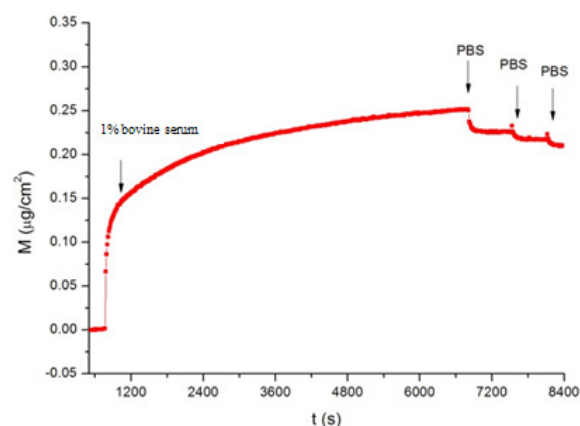


Figure 6.

Serum adsorption monitored by OWLS using the developed sample handling system.

Nanoparticle self assembly monitored by OWLS

The kinetics of assembly of polyethylene glycol (PEG)-coated superparamagnetic Fe₃O₄ nanoparticles in aqueous suspension on planar Si(Ti)O₂ surfaces have been determined using high-resolution Optical Waveguide Lightmode Spectroscopy (OWLS). Analysis of the results revealed that the initially uniform population was spontaneously transformed into two types of particles with significantly different adsorption behavior. The results were published in the *Journal of Nanoparticle Research*.

Self assembly of filamentous proteins

OWLS and ellipsometry was applied to study the self-assembly process of bacterial filaments on various model surfaces. The kinetics of adsorption and the structure of the layers were investigated. We have found that this filamentous protein forms a three dimensional film, in contrast to the two dimensional protein monolayers usually applied in biosensing experiments. The results were published in *Sensor Letters*.

Surface adhesion of living cells

Planar optical waveguides offer an ideal substratum for cells on which to reside. The materials from which the waveguides are made—high refractive index transparent dielectrics—correspond to the coatings of medical implants (e.g., the oxides of niobium, tantalum, and titanium) or the high molecular weight polymers used for culture -asks (e.g., polystyrene). The waveguides can furthermore be modified both chemically and morphologically while retaining their full capability for generating an evanescent optical field that has its greatest strength at the interface between the solid substratum and the liquid phase with which it is invariably in contact (i.e., the culture medium bathing the cells), decaying exponentially perpendicular to the interface at a rate controllable by varying the material parameters of the waveguide. Analysis of the perturbation of the evanescent field by the presence of living cells within it enables their size, number density, shape, refractive index (linked to their constitution) and so forth to be determined, the number of parameters depending on the number of waveguide lightmodes analyzed. No labeling of any kind is necessary, and convenient measurement setups are fully compatible with maintaining the cells in their usual environment. If the temporal evolution of the perturbation is analyzed, even more information can be obtained, such as the amount of material (microexudate) secreted by the cell while residing on the surface. Separation of parallel effects simultaneously contributing to the perturbation of the evanescent field can be accomplished by analysis of coupling peak shape when a grating coupler is used to measure the propagation constants of the waveguide lightmodes.

Measuring the surface adhesion of cells a dedicated optical instrument was developed and several collaborations were established with biophysicists and biochemists in order to monitor cell adhesion on chemically modified surfaces. The modeling of the adhesion and spreading kinetics of living cells is an important part of our research.

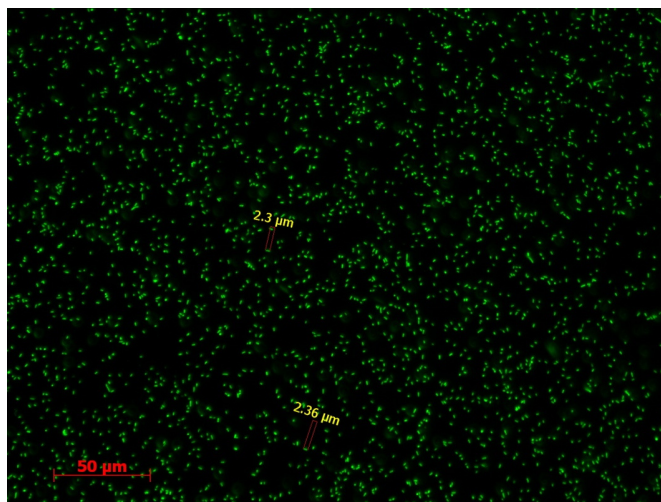


Figure 7.

Adhered bacterial cells on the surface of the biosensor. Investigating the biosensor surface by a microscope after bacterial adhesion the biosensor signal can be calibrated.

Functional nanocomposite biological films

The self-assembly of flagellin–polyelectrolyte multilayer films were studied using OWLS, confocal microscopy and AFM. It was found that flagellin can be incorporated into the polymer layer.

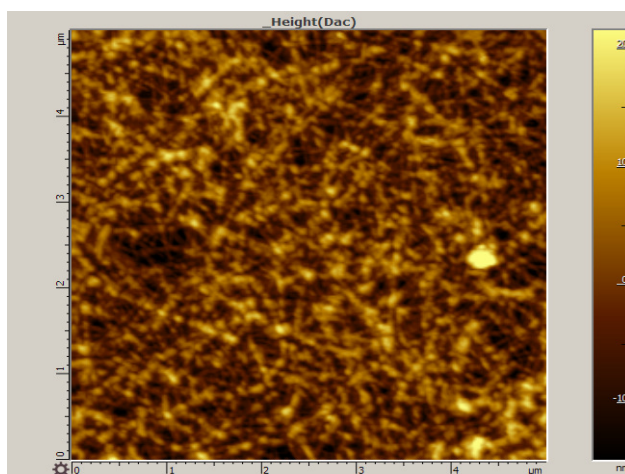


Figure 8.

AFM image of the flagellin-polyelectrolyte nanocomposite film. Remarkably, the flagellin self-assembled into filaments in the polyelectrolyte itself.

Using genetic engineering the flagellin's D3 domain can be modified in order to have receptor or enzymatic properties. Thus, these findings open the way for developing flagellin based functional films. Interestingly, the flagellin self-assembled into filaments in the polyelectrolyte layer itself (see **Fig. 8.**). This is most probably due to the stabilization of the disordered terminal helices by the positively charged polyelectrolyte. We have also found that some of these films are significantly reducing the surface adhesion of bacterial cells. More detailed research is needed to be able to publish the results, but several high quality publications can be expected from these novel directions.

Exploitation possibilities of the project results

There are several novel ideas in the project which could be patented. But, to be able to decide, further scientific investigation and careful economical considerations are needed. These ideas are connected to the novel waveguide sensors developed, but the interesting findings about the functional polyelectrolyte-flagellin nanocomposite films (especially their novel fabrication route) might be also patentable. Also, using self-assembled and oriented flagellin layers as antibacterial coatings is an interesting novel idea, which could be considered as a basis of a patent application.

Supervised students

The present project gave the possibility for several BSc, MSc and PhD level students to join to these research directions. The names of the students, their research topic with the main outcome are listed below:

1. *Péter Kozma* (PhD student at Pannon University): Biosensor development
Peter will defend his Thesis soon. Part of the thesis dealing with ellipsometric measurements, which was supervised by Dr. Peter Petrik.
2. *Dániel Patkó* (PhD student at Pannon University): Biosensor development and its application
Daniel successfully finished the 1st year of his PhD studies.
3. *Noémi Kovács* (MSc student at Technical University): Protein adsorption characterized by optical biosensors
Noémi will defend her MSc Thesis in January 2012. She also wrote a Student Work Report (TDK in Hungarian) from these works in 2010, which was considered at the National Conference.
4. *Norbert Orgován* (MSc student at Eötvös University): Fluid handling in biosensor experiments and monitoring living cells by label-free optical biosensors.

Norbert submitted two TDK works which received 2nd and 3rd price at the TDK Competition of the Eötvös University in 2010 and 2011, respectively. Both works were and will be considered at the National Conference.

5. *Juhász Krisztina* (MSc student at Eötvös University): Biosensing experiments using optical waveguides. The surface chemistry part of the work was supervised by Dr. Sándor Kurunczi.

A TDK report was submitted (jointly with Norbert Orgován), which received 3rd price at the TDK Competition of the Eötvös University in 2011 (see also above).

6. *Enikő Farkas* (BSc student at Technical University): Functional nanocomposite films
A TDK work was submitted.

7. *Kovács Boglárka* (BSc student at Technical University): bacterial adhesion
A TDK work and a BSc Thesis was submitted. The TDK work received 3rd price at the Technical University.

8. *Ádám Horváth* (BSc student at Pázmány University): Program development for analyzing cell adhesion data in Matlab environment.
A BSc Thesis was submitted.

9. *Balázs Kobzi* (BSc student at Eötvös University): Lipid bilayers on biosensors
A TDK work was submitted. The main supervisor was Prof. Éva Kiss.