Project No: 231055
Project Acronym: OPTIBIO

Optical resonances for label-free biosensing

ERG report covering the whole project

Robert Horvath

1. Publishable summary

In the framework of the Marie Curie Reintegration Project Robert Horvath joined the Research Institute of Technical Physics and Materials Science (MFA) and could return work in his home country, Hungary, continuing the momentum built up by 7 years of very fruitful research in Denmark and in England. At the host institute a long term strategic plan is to build up a strong research direction in the field of bio- and chemical sensing and to conduct basic and applied research in these fields. The reintegrated researcher significantly strengthened this direction with his relevant experiences in the field of optical waveguide based biosensors and their applications. Particularly, during the project novel optical waveguide based sensors were developed for label-free sensing of biomolecules and living cells. A new sensing concept, Grating Coupled Interferometry, was introduced for high resolution and cost-effective label-free sensing. Planar optical sensors were also used to monitor the surface adhesion of proteins, nanoparticles and the surface assembly of flagellin based films. Theoretical modeling of these resonator structures were also investigated, mainly focusing on anisotropic effects in the deposited layers. Using the fellowship Robert Horvath started to create his own research group by building up a new laboratory and supervising nine BSc, MSc or PhD level students in the above-mentioned scientific topics. After the fellowship, he was granted a 5 years "Lendület" career development grant by the Hungarian Academy of Sciences fully completing his reintegration in Hungary.

2. Project objectives

2.1. Scientific objectives

(The original scientific objectives copy and pasted from the project application.)

"The present project aims to develop novel optical waveguide based sensors capable of supporting more detailed information about the structure of thin films or living cells with increased sensitivity in a label-free manner. This is achieved by designing and fabricating

novel reverse and conventional waveguide configurations supporting several modes and using different excitation wavelengths. Especially, novel Si (using infrared wavelengths) and metal-oxide processing technology available at the host institute will be explored and combined with the applicants experience in soft lithography. The fabricated sensors will be applied to test and develop the above mentioned novel sensor matrix based on functionalized protein assemblies for detecting small analytes and living cells. To increase sensitivity for small analytes the sensing matrix will be assembled as thick films for example by using polyeletrolytes to deposit several hundreds of single sensing layers on top of each other. In case of cell sensing the flagellar filaments are applied as a single layer or deposited perpendicularly to the surface of the sensor. Theoretical modelling of the fabricated structures and their sensing capabilities will be an important part of the research, especially modelling analyte layer anisotropy and inhomogeneity building on the applicant experience in these fields. The knowledge obtained in this way will help to find optimum sensor configurations and will future extend the capabilities of the technique in both basic and applied research on biological films or living cells; or as a general platform for nanotechnological research."

2.2. Other objectives

Further important objectives of the Marie Curie Reintegration Fellowships are to help the long-lasting career development of the researcher, transfer his previously acquired knowledge to the host institute, to help in the development of scientific and industrial co-operations.

3. Achievements towards the objectives

3.1. Major research findings with initiated new directions

Development of biosensor instrumentation

An interferometric sensor based on gratings on a planar optical waveguide is introduced in collaboration with Creoptix GmbH. 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.

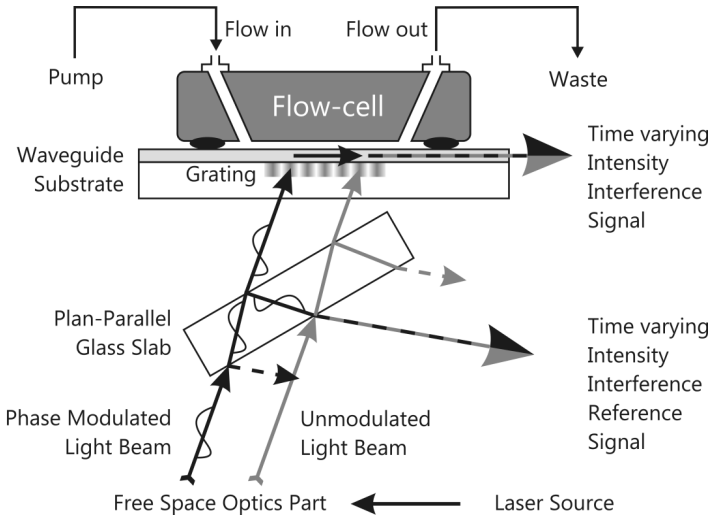

Figure 1.

Cross section of the developed single channel biosensor device.

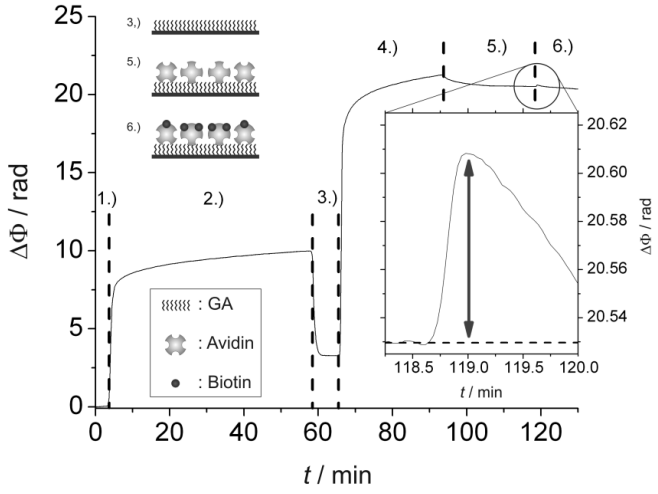

Figure 2.

Demonstration of biotin binding to an immobilized avidin layer. The expariment demonstartes 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.

One publication is under preparation at the moment 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.

I have also built a high resolution goniometer based setup capable of monitoring a wide range of waveguide parameters and capable to measure the modes of multimode or metal-clad waveguides too. Building on the expertise of MFA we mainly fabricated tantalium-pentoxide waveguides with ion implanted diffractive optical elements for biosensor applications. We have also started a research line on waveguide fabrication with imprinted polymer nanostructures. A BSc thesis was successfully submitted based on this work.

Flagellin adsorption monitored by optical waveguides

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

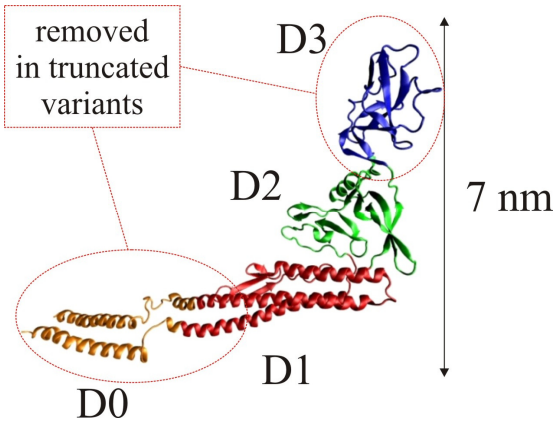

Figure 3.

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 ΔD3_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.**).

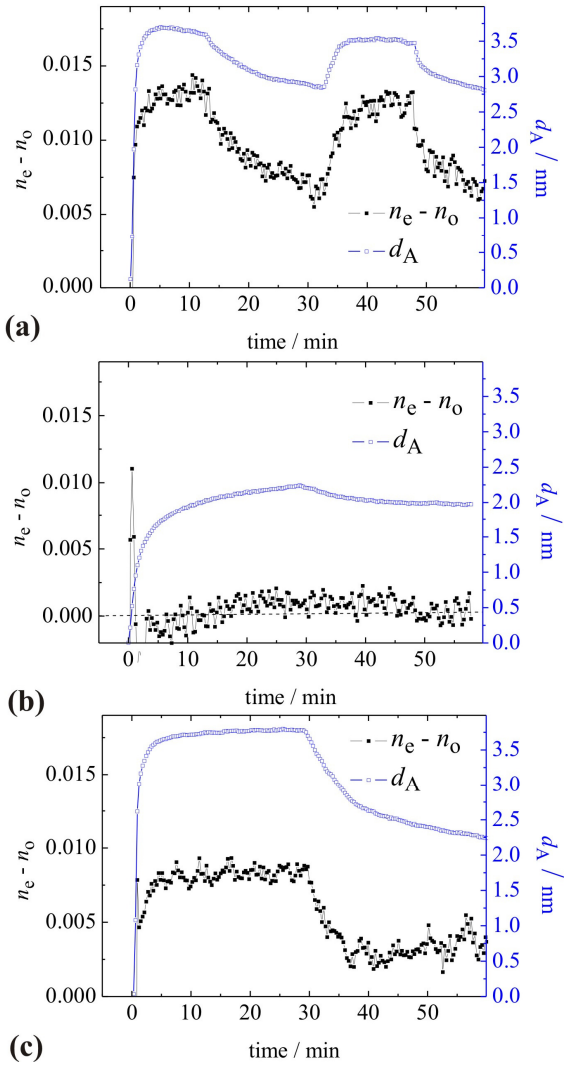

Figure 4.

Birefringence and averaged thickness of the adsorbed layers treating them as uniaxially anisotropic. ***(a)*** *flagellin,* ***(b)*** *F40 and* ***(c)*** *ΔD3_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 to be submitted to *Analytical Chemistry* or to *Langmuir*.

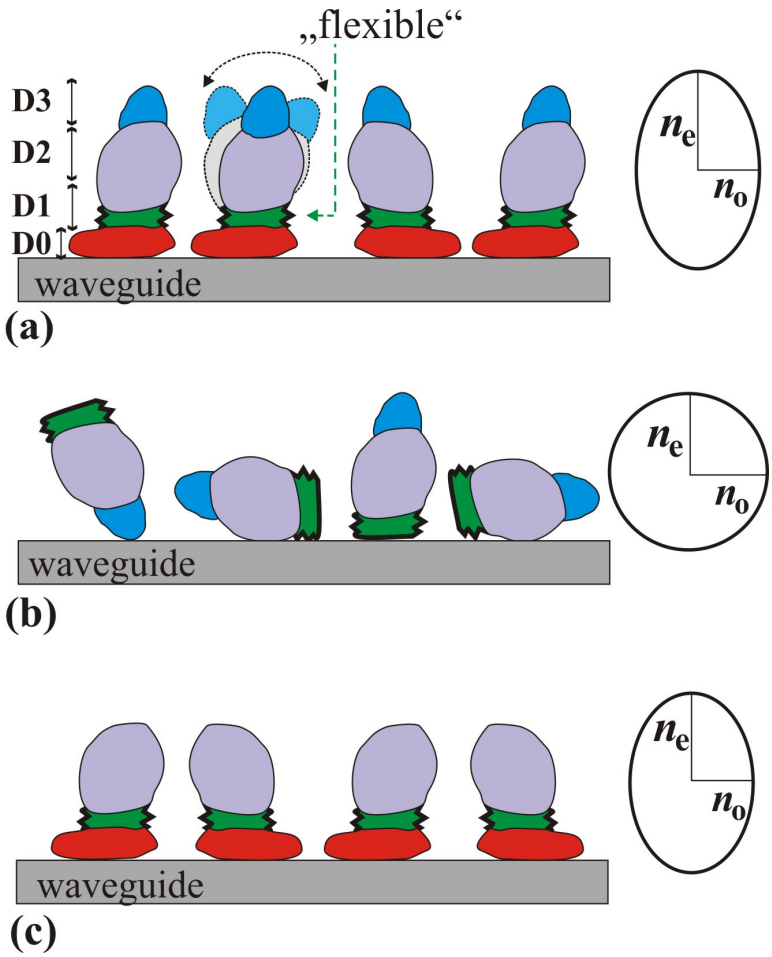

Figure 5.

Schematic illustration of the internal structure of the adsorbed protein layers. ***(a)*** *flagellin* ***(b)*** *F40 and* ***(c)*** *ΔD3_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.*

Lipid bilayers monitored by optical waveguides

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. Until now, a TDK work was submitted.

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 signifocant 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 under preparation.

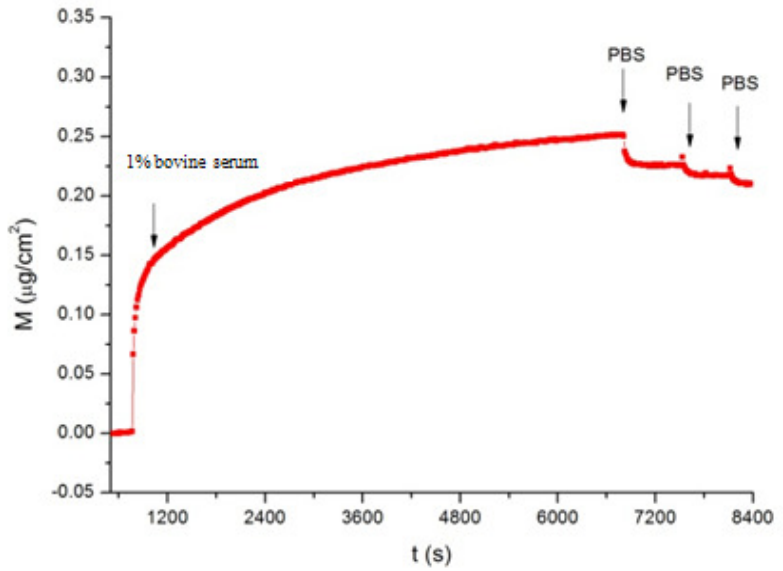

Figure 6.

Serum adsorption monitored by OWLS using the developed sample handling system using a septum based injecor.

Nanoparticle adhesion monitored by OWLS

The kinetics of assembly of polyethylene glycol (PEG)-coated superparamagnetic Fe_3O_4 nanoparticles in aqueous suspension on planar $Si(Ti)O_2$ 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* and in the *Journal of Chemical Physics*.

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 living cells a dedicated optical instrument was developed and several collaborations were established with biophysicists and biochemists (for example with Dr. Bálint Szabó and Prof. Gábor Mező at Eötvös University) in order to monitor cell adhesion on chemically modified surfaces (matrix proteins, mucins, polymers grafted with cyclopeptides developed by Prof. Mező etc.). The modeling of the adhesion and spreading kinetics of living cells is an important part of our research and several publications were already accepted from this topic (*see list of publications*). There is a significant transfer of knowledge in this regard since this scientific topic was completely missing at the host institute.

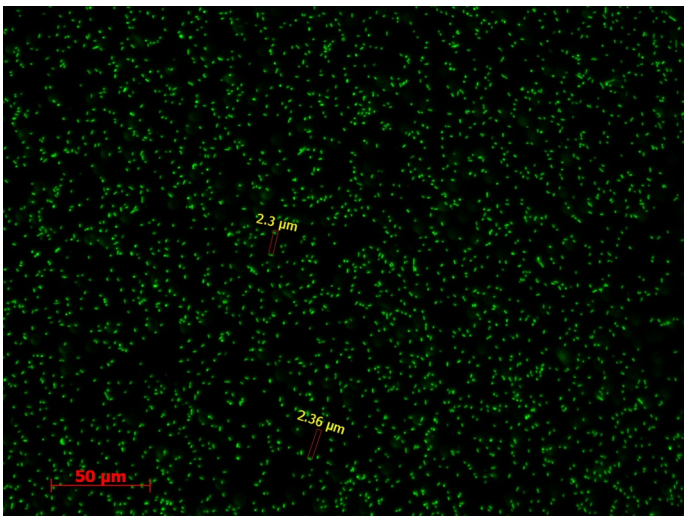

Figure 7.

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 could be incorporated into the polymer layer. 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. TDK works and BSc, MSc Theses were submitted from these results and the key publications are under preparation.

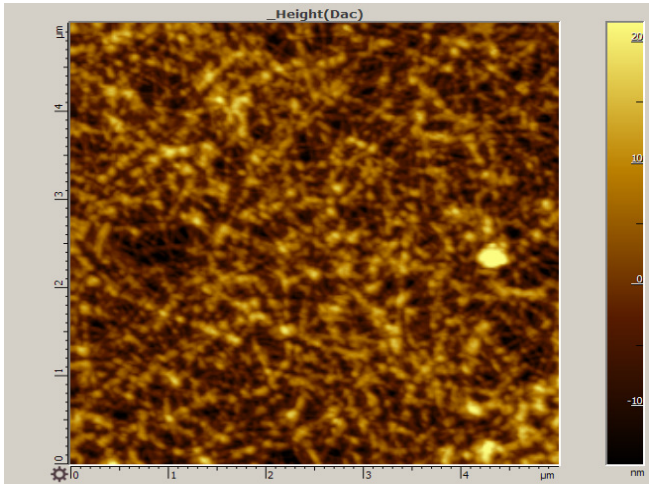

Figure 8.

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

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. In addition, 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.

3.2. Dissemination activities

(list of publications and presentations)

Robert Horvath
Evanescent optical waves for label-free monitoring of live cell status and behavior
2011 IEEE Photonics Society Summer Topical Meeting Series , art. no. 6000051 , pp. 73-74 (2011)

Patkó Dániel, Kaspar Cottier, Hámori András, Horváth Róbert
Jelölésmentes interferometrikus optikai bioszenzor referencia csatornával

A mi világunk kémiája Budapest 2011 (2011)

Patko D, Hamori A, Cottier K, Kurunczi S, Horvath R
Label free biosensing using Grating Coupled Interferometry
In: 8th EBSA European Biophysics Congress..
Budapest: Springer, LNCS 2098, pp. 230-231.(ISBN:(ISBN:ISSN: 0175-7571))

Orgován Norbert, Patkó Dániel, Kovács Noémi, Kurunczi Sándor, Horváth Róbert
Folyadékkezelés OWLS kísérletekben
A mi világunk kémiája Budapest 2011 (2011)

Orgován Norbert, Horváth Róbert
Élo sejtek jelölésmentes vizsgálata optikai bioszenzorral
A mi világunk kémiája Budapest 2011 (2011)

N Orgovan, D Patko, N Kovacs, S Kurunczi, R Horvath
Fluid handling in biosensor experiments: OWLS fluidics
EuroNanoForum Budapest 2011 (2011)

N Kovacs, D Patko, N Orgovan, S Kurunczi, A Vardai, A Muskotal, F Vonderviszt, R Horvath
Adsorption of flagellin based protein layers
EuroNanoForum Budapest 2011 (2011)

Kozma P, Kozma D, Nemeth A, Jankovics H, Kurunczi S, Horvath R, Vonderviszt F, Fried M, Petrik P
In-depth characterization and computational 3D reconstruction of flagellar filament protein layer structure based on in situ
spectroscopic ellipsometry measurements
APPLIED SURFACE SCIENCE 257:(16) pp. 7160-7166. (2011)

Kozma P, Hamori A, Kurunczi S, Cottier K, Horvath R
Grating coupled optical waveguide interferometer for label-free biosensing
SENSORS AND ACTUATORS B-CHEMICAL 155:(2) pp. 446-450. (2011)

Kovács Noémi, Patkó Dániel, Orgován Norbert, Kurunczi Sándor, Várdai Attila, Muskotál Adél, Vonderviszt Ferenc, Horvath Robert
Flagellin alapú fehérje rétegek felületi adszorpciója
A mi világunk kémiája Budapest 2011 (2011)

Horváth Róbert
Optikai bioszenzorok és alkalmazásaik
A mi világunk kémiája, Budapest 2011 (2011)

Dortu F, Egger H, Kolari K, Haatainen T, Furjes P, Fekete Z, Bernier D, Sharp G, Lahiri B, Kurunczi S, Sanchez JC, Turck N, Petrik P, Patko D, Horvath R, Eiden S, Aalto T, Watts S, Johnson NP, De La Rue RM, Giannone D
Design and process development of a photonic crystal polymer biosensor for point of care diagnostics

In: Ramanujam N, Popp J
CLINICAL AND BIOMEDICAL SPECTROSCOPY AND IMAGING II.
Berlin: SPIE - The International Society for Optical Engineering, pp. 1-11.

D Patko, K Cottier, A Hámori, R Horvath
Label-free optical biosensor with improved stability and sample handling
EuroNanoForum Budapest 2011 (2011)

D Patko, K Kolari, S Kurunczi, T Aalto, R Horvath
Compact Atomic Layer Deposited Overlayers to Increase Biosensor Stability
EuroNanoForum Budapest 2011 (2011)

Ansari F, Kavosh M, Horvath R, Ramsden JJ
Particle speciation during PEG-Fe(3)O(4) hybrid nanoparticle self-assembly on Si(Ti)O(2)
JOURNAL OF NANOPARTICLE RESEARCH 13:(1) pp. 193-198. (2011)

Robert Horvath
Optical Waveguide Biosensors for Proteins and Cells: Invited presentation at ICOOPMA, Budapest
Fourth International Conference on Optical, Optoelectronic and Photonic Materials and Applications, Budapest (2010)

Robert Horvath
Optical Resonances with Tunable Probing Volume for Label-free Biosensing
ETH Zurich - Bioplasmonics 2010 (2010)

Nemeth A, Kozma P, Hulber T, Kurunczi S, Horvath R, Petrik P, Muskotal A, Vonderviszt F, Hos C, Fried M, Gyulai J, Barsony I
In Situ Spectroscopic Ellipsometry Study of Protein Immobilization on Different Substrates Using Liquid Cells
SENSOR LETTERS 8:(5) pp. 730-735. (2010)

Kovacs N, Orgovan N, Kurunczi S, Horvath R, Vonderviszt F, Ramsden JJ
Flagellin based protein layers for biosensing
3rd International NanoBio Conference, August 24-27, 2010; ETH Zurich (2010)

Kovacs N, Orgovan N, Kurunczi S, Vonderviszt F, Horvath R
Flagellin adsorption on model surfaces
CIMST Interdisciplinary Summer School on Biomedical Imaging, September 6-17, 2010; ETH Zurich (2010)

Ansari Farahnaz, Kavosh Masoud, Horvath Robert, Ghalamboran Mohammad Reza, Ramsden Jeremy J
Bacterial Adsorption Onto Monolayer Ferromagnetic Nanofilms
JOURNAL OF BIONANOSCIENCE 4:(1-2) pp. 119-122. (2010)

Amirreza Aref, Robert Horvath, Jeremy J Ramsden
Spreading kinetics for quantifying cell state during stem cell differentiation
JOURNAL OF BIOLOGICAL PHYSICS AND CHEMISTRY 10:(4) pp. 145-151. (2010)

Aalto Timo, Giannone D, Horvath R, Sanchez J-C, Johnson N, Eiden S, Watts S
Medical diagnostics, microfluidics, integrated optics and materials science being merged in the P3SENS project: 1st International Scientific Conference: Microfluidics in Bioanalytical Research and Diagnosis. Espoo, September 30 - October 1, 2010 Abstract Book. VTT. Helsinki (2010), p. 47

Zourob Mohammed, Skivesen Nina, Horvath Robert, Mohr Stephan, Goddard Nicholas J
Deep-Probe Optical Waveguides for Chemical and Biosensors: Advanced Photonic Structures for Biological and Chemical Detection
In: Fan Xudong
Advanced Photonic Structures for Biological and Chemical Detection
New York: Springer, 2009. pp. 395-441.
(Integrated Analytical Systems)
(ISBN:978-0-387-98063-8)

Ramsden JJ, Horvath R
Optical biosensors for cell adhesion
JOURNAL OF RECEPTOR AND SIGNAL TRANSDUCTION RESEARCH 29:(3-4) pp. 211-223. (2009)

McColl J, Horvath R, Aref A, Larcombe L, Chianella I, Morgan S, Yakubov GE, Ramsden JJ
Polyphenol Control of Cell Spreading on Glycoprotein Substrata
JOURNAL OF BIOMATERIALS SCIENCE-POLYMER EDITION 20:(5-6) pp. 841-851. (2009)

Kurunczi S, Horvath R, Yeh YP, Muskotál A, Sebestyén A, Vonderviszt F, Ramsden JJ
Self-assembly of rodlike receptors from bulk solution.
JOURNAL OF CHEMICAL PHYSICS 130: p. 011101. (2009)

Kozma P, Hamori A, Cottier K, Kurunczi S, Horvath R
Grating coupled interferometry for optical sensing
APPLIED PHYSICS B - LASERS AND OPTICS 97:(1) pp. 5-8. (2009)

Aref A, Horvath R, McColl J, Ramsden JJ
Optical monitoring of stem cell-substratum interactions
JOURNAL OF BIOMEDICAL OPTICS 14:(1) Paper 010501. (2009)

Aggarwal N, Lawson K, Kershaw M, Horvath R, Ramsden J
Protein adsorption on heterogeneous surfaces
APPLIED PHYSICS LETTERS 94:(8) Paper 083110. (2009)

3.3. Student supervision

I am quite happy to note that a significant number of students are interested in biosensing and in nanobiotechnology in Hungary. Instead of working alone I have spent a massive amount of my time with *recruiting students and transfer my existing knowledge to them*. The more senior staff at MFA is supportive in this regard. During my years abroad, I did not have the possibility to officially have my own students, even if I spent a significant amount of my time with supervising, training and helping students. Now the situation is dramatically changed, I have successfully involved several undergraduate students into my research work from the Eotvos University and from the Technical University of Budapest, Pannon University etc. Two PhD students were also working under my supervision, developing advanced biosensor interrogation techniques based on optical interferometry. For example, Peter Kozma (already a postdoc in Germany) participated on the 2009 EMRS conference and received a special price with his work. This success was even spotted at the web page of the Hungarian Academy of Sciences.

The present project gave the possibility for several BSc, MSc and PhD level students to join to these novel 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 defended his Thesis. 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 defends 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 and a BSc thesis 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.

4. General conclusions and further impact on the career of the researcher

The Marie Curie Reintegration Fellowship is hosted by the Research Institute for Technical Physics and Materials Science (in Hungarian MTA MFA), Budapest where basically we started to set up a new laboratory, the so-called Nanosensorics Laboratory, focusing on the development and applications of optical biosensors. The fund was a great help to me to start to *start my work* and to try to initiate new research directions. Therefore, the resources I mainly used to buy basic small equipments and consumables for the project (optical holders, tables, lamps, refracrometer, chemicals etc.). Unfortunately, I quickly realized that the ERG funding is quite limited, so I also started to apply for additional funding from the European Commission (ICT, Starting Independent Research Grant) and Hungarian Funding Agencies (OTKA, NKTH).

In summary, the reintegration of Robert Horvath was highly successful which is also recognized by a "Lendület" career development grant received after the ERG fellowship period. Long lasting scientific collaborations were also established with Hungarian researchers and several students could benefit from the reintegration of Robert Horvath.