NKFI-ID: 117755 Type: ERC_HU_15

PROJECT FINAL REPORT

Label-free optical biosensors for living cell research

PI: Robert Horvath

Summary of project results

The present research focused on single cell analyzis using label-free optical biosensors. The main aim was to build up the basis of relevant methodologies to be able to sense refractive index variations inside single living cells and to manipulate individual cells. Molecular movements, adhesion events were characterized with significant relevance to biomedical and health related research and development. The proposed research plan realized parts of the ERC project submitted by Robert Horvath, which received category "A". The present project significantly strengthened the ongoing Momentum (Lendület) research activity, too.

Using the valuable funds, two important instruments were set up in the Nanobiosensorics Laboratory (*i*) a computer controlled micropipette and (*ii*) a plate based biosensor using the resonant waveguide technology with 22 micron lateral resolution.

Using a computer controlled micropipette we developed a methodology to pick up individual cells from suspension. This result will be further developed to deposit these individual cells onto biosensor surfaces for further measurements. The high lateral resolution biosensor was tested with 9 micron sized microbeads, and this methodology was further extended to living individual cells.

Cell adhesion measurements using the micropipette and its comparison with the data of label-free optical biosensors were - as planned - also performed. These results are well documented in the published scientific publications. Cancer cells and immune cells were investigated in close collaboration with the group of Prof. Anna Erdei (ELTE).

We also paid special attention to monitor the biophysical characteristics of cells upon exposure to natural compounds (like EGCG). One review paper and two journal publications resulted from this research line.

We have introduced our biosensors in a completely new field, to monitor molecular movement inside living cells upon external stimuli. One journal publication (Sensors and Actuators B) summarizes these novel results.

Concerning the coatings of the biosensor chips - as planned - dextran and flagellin based coatings were developed to control cell adhesion and to decrease nonspecific binding on the surface of the biochips. Two publications in highly ranked journals summarize these results.

In summary, important equipment were installed and in several planned research directions in the label-free biosensing filed high impact papers were published (see the attached list of papers) or are under publication.

The detailed results of the project are summarized below.

Biophysical characteristics of proteins and living cells exposed to the green tea polyphenol epigallocatecin-3-gallate (EGCg): Review of recent advances from molecular mechanisms to nanomedicine and clinical trials

Traditionally, tea was drunk to eliminate toxins, to improve blood flow and resistance to diseases, so its habitual consumption has long been associated with health benefits. Among natural compounds and traditional Chinese medicines, the green tea polyphenol epigallocatechin gallate (EGCg) is one of the most studied active substance. Tea catechins, especially (-)-EGCg, have been shown to have various health benefits, for example anti-metastasis, anti-cardiovascular, anti-cancer, antiinflammatory and antioxidant effects (Fig. 1). In 2015 we focused on the molecular scale interactions between proteins and EGCg with special focus on its limited stability and antioxidant properties, the observed biophysical effects of EGCg on 112 various cell lines and cultures. The alteration of cell adhesion, motility, migration, stiffness, apoptosis, proliferation, as wells as the different impacts on normal and cancer cells are all summarised in our prospective review article [1]. We also handled the works performed using animal models, microbes and clinical trials. Novel ways to develop its utilization as therapeutic purposes in the future are discussed too, for instance, using nanoparticles and green tea polyphenols together to cure illnesses, and the combination of EGCg and anticancer compounds to intensify their effects. In this review we summarize the experiments and results of the past few years. The limitations of the employed experimental models and the criticisms on the interpretation of the obtained experimental data are summarized as well. We also pointed out some inaccuracies in the literature.

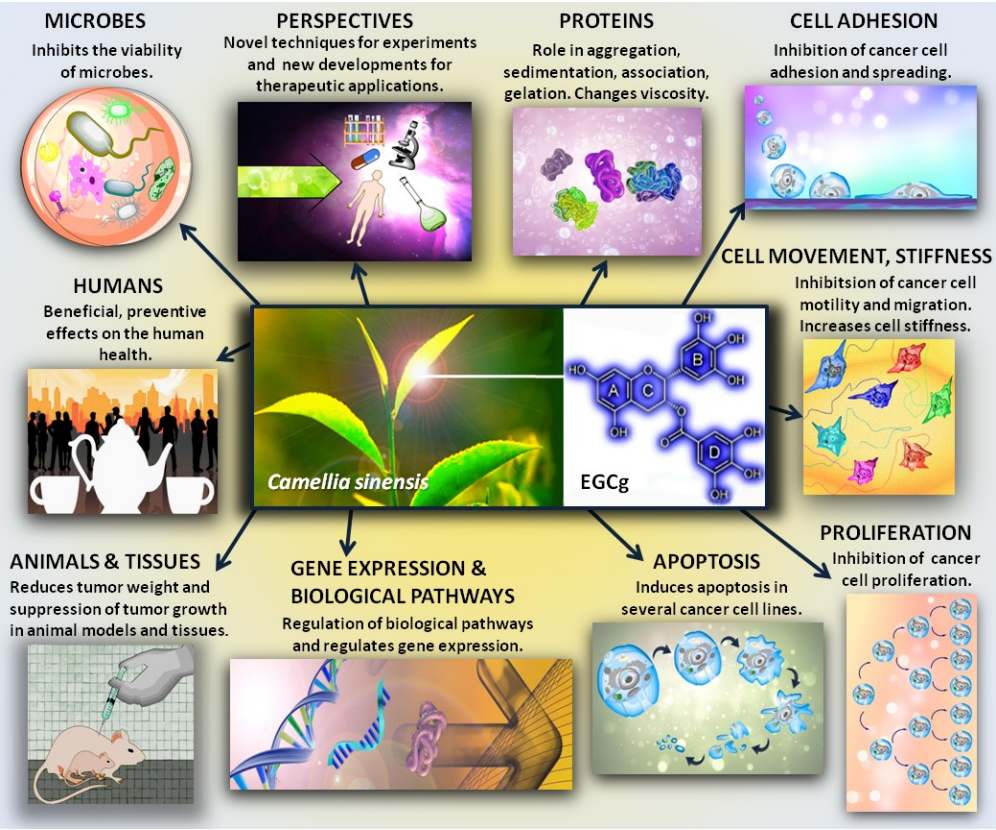

Figure 1 *Diversified effects of EGCg.*

[1] B. Peter, Sz. Bősze, R. Horváth,. Biophysical characteristics of proteins and living cells exposed to the green tea polyphenol epigallocatechin-3-gallate (EGCg): review of recent advances from molecular mechanisms to nanomedicine and clinical trials. *European Biophysics Journal* (46) 1, 1-24. (2017)

Green tea polyphenol tailors cell adhesivity of RGD displaying surfaces: multicomponent models monitored optically

A wide range of experimental methods are available to measure cell adhesion and cell–surface interacions, however, most of them have serious disadvantages when a multicomponent model of cell adhesion has to be quantitatively investigated in a reasonable time frame. (For example, labeling techniques use fuorescent markers that may afect normal cell behavior and the imaging time is often limited by the bleaching of the marker). In contrast, we highlighted the remarkable potentials of high-throughput resonant waveguide gratings in studying multicomponent model systems of cell–surface interactions. The interaction of the anti-adhesive, antifouling coating, PLL-g-PEG and its RGD (Arg-Gly-Asp) functionalized form, PLL-g-PEG-RGD, with the green tea polyphenol, EGCg was in situ monitored. Right after, cellular adhesion on the EGCg exposed coatings was recorded in real-time (Fig.1). The plate based sensor configuration allowed following the above processes with different surface coatings, EGCg states and concentrations in a single run, on the same biosensor plate. Despite the reported excellent antifouling properties of the above polymer coatings, EGCg strongly interacted with them, and affected their cell adhesivity in a concentration dependent manner. The differences between the effects of the freshly prepared and oxidized EGCg solution could be also first demonstrated. The measured interactions were signifcantly stronger for the oxidized EGCg solution, highlighting the importance of storage conditions of EGCg solutions, often overlooked in present literature. Using a semiempirical quantumchemical method we showed that EGCg binds to the PEG chains of PLL-g-PEG and PLL-g-PEG-RGD by hydrogen bonds. Moreover, the calculations illuminated the diferences in binding afnity between the fresh and oxidized EGCg, well supporting the experimental findings. Our work lead to a new model of polyphenol action on cell adhesion ligand accessibility and matrix rigidity [1].

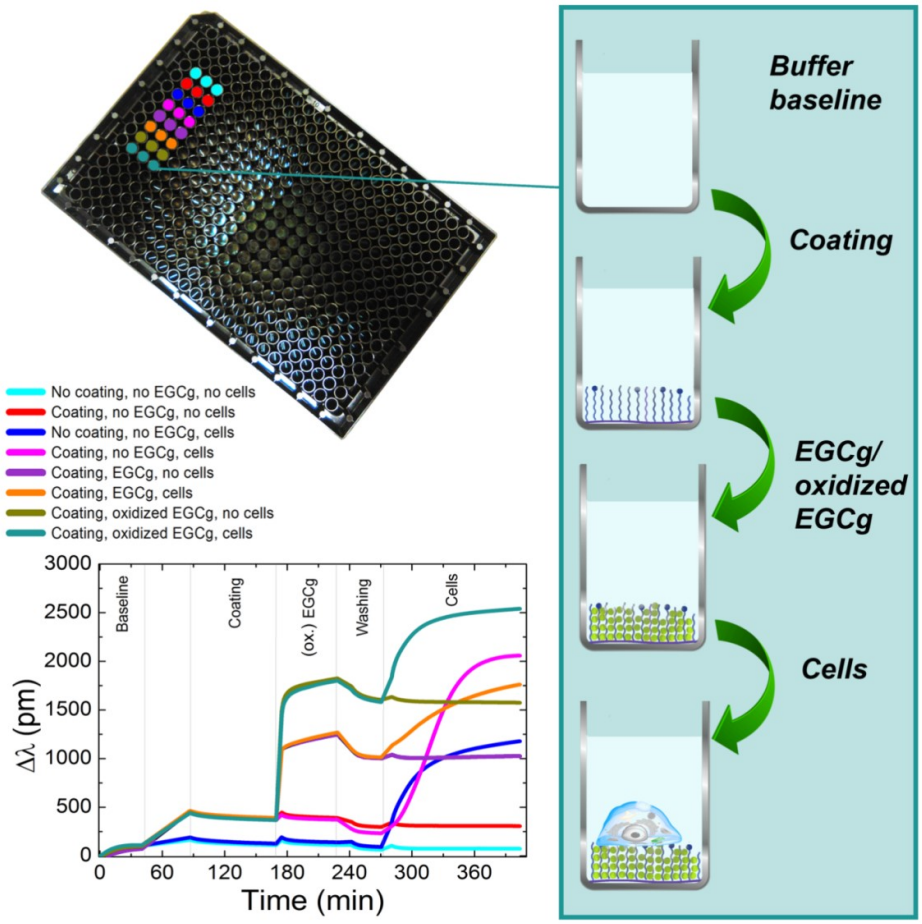

Figure 1 *In situ kinetic curves recorded by the Epic BT instrument. A 384-well plate used in the experiment is also shown, together with the manipulation steps in a typical well (right scheme). Typical experimental curves are plotted (bottom left corner) for PP:PPR coating, and 500 µg/ml EGCg concentration. The detailed experimental conditions corresponding to the various kinetic curves are indicated above the graph.*

[1] B. Peter, E. Farkas, E. Forgacs, A. Saftics, B. Kovacs, S. Kurunczi, I. Szekacs, A. Csampai, Sz. Bosze, R. Horvath. Green tea polyphenol tailors cell adhesivity of RGD displaying surfaces: multicomponent models monitored optically. *Scientific Reports* 742220 (2017)

Label-free optical biosensor for on-line monitoring the integrated response of human B cells upon the engagement of stimulatory and inhibitory immune receptors

The majority of current cell-based assays relies on the measurement of a single event at a predetermined time point in a specifically chosen signaling path-way, let it be second messenger release, reporter-gene production or target translocation. These measurements require the use of labeled compounds, sometimes the modification of cells to express the target in larger amount or to produce a reporter molecule to be able to monitor receptor engagement [1]. The mentioned manipulations can be toxic for the cells and can interfere with normal cellular physiology of the target receptors or their environment and the applied fluorescence and colored compounds may induce elevated background [1]. Consequently, functional cellular assays which can report from different signaling events in real time without the application of molecular engineering (in providing the suitable cellular partner) and without the use of labelling would be of high value for both theoretical and practical studies even if they are more complex and less specific than cell-based biochemical assays.

To be able to obtain holistic pictures about B cell responses to complex interlocking stimulations Epic BT optical biosensor was applied and set to establishing the method using human B cell lines, derived from Burkitt's lymphomas. We successfully immobilized non-adherent B cells on the surface of the biosensors, without the ligation of any specific receptors or adhesion molecules. This way we were able to demonstrate that engagement of the antigen specific B cell receptors (BCR) induced reproducible dynamic mass redistribution (DMR) inside the cells as a measure of receptor activation (see Fig.1). The initiated DMR response proved to be specific, since only antibodies recognizing the BCR could generate the response; neither the assay-buffer, nor high concentration of indifferent proteins or non-specific antibodies had any effect. The measure of cell activation was sensitive, concentration dependent, and specifically and dose-dependently inhibited by the Syk inhibitor BAY 61-3606. The BCR-triggered DMR response was evoked from three human Epstein-Barr virus (EBV) negative B cell lines, but could not be elicited in two EBV-positive BL cell lines, where the presence of the EBV-derived LMP2A protein desensitizes the cells' response to the BCR-induced signaling.

Therefore, our work opens new avenues to study complex signaling events and to decipher interactions within the signaling network during B cell activation [1].

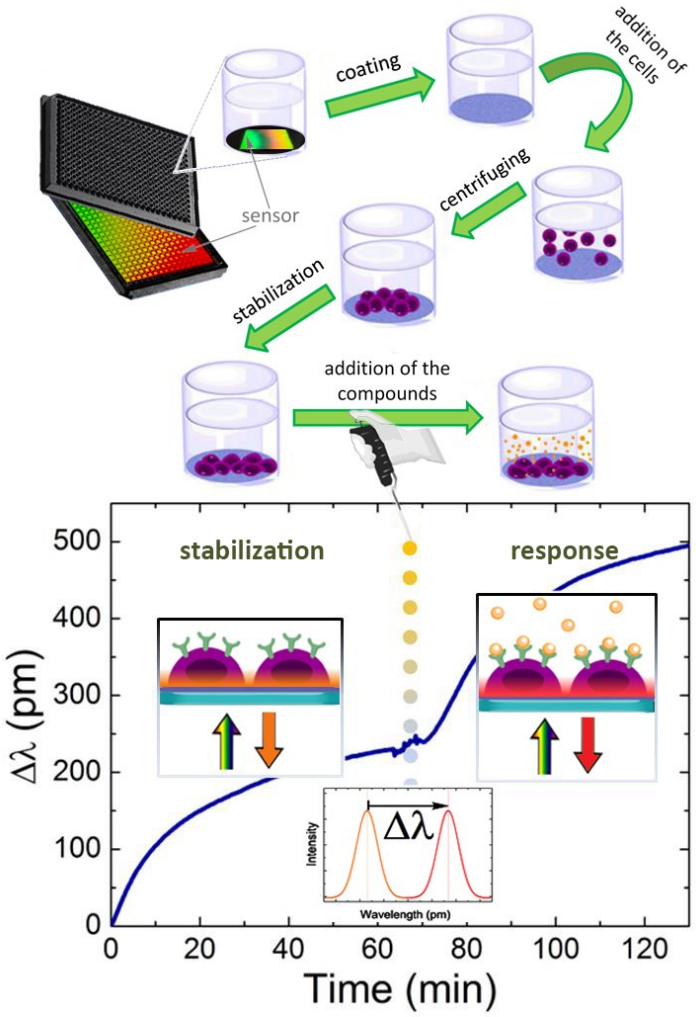

Figure 1 *Experimental setup to perform real-time measurement of dynamic mass redistribution (DMR) using B-cell lines (A); and schematic illustration of the principle of DMR detection (B)*[1].

[1] I. Kurucz, B. Peter, A. Prosz, I. Szekacs, R. Horvath, A. Erdei. Label-free optical biosensor for on-line monitoring the integrated response of human B cells upon the engagement of stimulatory and inhibitory immune receptors. *Sensors and Actuators B*, 240, 528–535 (2017)

ZnO nanostructure templates as a cost-efficient mass-producible route for the development of cellular networks

The development of artificial surfaces which can regulate or trigger specific functions of living cells, and which are capable of inducing in vivo-like cell behaviors under in vitro conditions has been a long-sought goal over the past twenty years. In our work, an alternative, facile and cost-efficient method for mass-producible cellular templates is presented. The proposed methodology consists of a cost-efficient, two-step, all-wet technique capable of producing ZnO-based nanostructures on predefined patterns on a variety of substrates. ZnO—apart from the fact that it is a biocompatible material—was chosen because of its multifunctional nature which has rendered it a versatile material employed in a wide range of applications. Si, Si_3N_4, emulated microelectrode arrays and conventional glass cover slips were patterned at the micrometer scale and the patterns were filled with ZnO nanostructures. Using HeLa cells, we demonstrated that the fabricated nanotopographical features could promote guided cellular adhesion on the pre-defined micron-scale patterns only through nanomechanical cues without the need for further surface activation or modification (see Fig. 1) [1]. The suggested methodology is extremely promising for the creation of engineered cellular networks through purely nanomechanical cues.

One of the most important results of this study was a better understanding of the dynamics of selective cellular adhesion and the implication of time as a controlling factor that must be combined with nanomechanosensation. Further studies are foreseen for the evolvement of the method into a technology that can be readily transferred to mass-production and the development of real-life viable products [1].

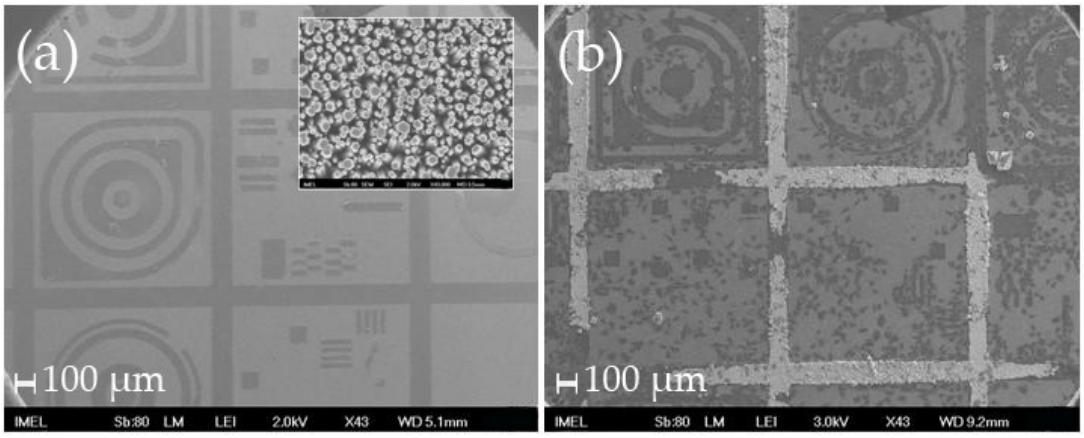

Figure 1 *SEM images of* ***(a)*** *of the patterned Si wafer with larger patterns and larger flat areas (the darker areas contain the ZnO nanorods shown in the zoom-in inset image; scale bar: 100 nm);* ***(b)*** *the same samples with HeLa cells after 4 days in culture, where one can see that the cells mostly adhere onto the flat areas (the white areas are the nanorods that have been covered by salts from the nutrient medium that was not fully removed after washing).*

[1] E. Makarona, B. Peter, I. Szekacs, C. Tsamis, R. Horvath. ZnO nanostructure templates as a cost-efficient mass-producible route for the development of cellular networks. *Materials,* 9 (4), 256 (2016)

Fabrication and characterization of ultrathin dextran layers: Time dependent nanostructure in agueous environments revealed by OWLS

(Hydrogel film fabrication for biosensing)

Fabrication of a stable and reproducible surface with the required chemical functions are one of the major challenge in the development of label-free biosensors. Polysaccharide dextran interface layers are able to improve the sensitivity of biosensors, owing to the anti-fouling property and the high receptor immobilization capacity of the dextran chains.

Carboxymethyl-dextran (CMD) was synthetized in our laboratory from the native dextran. Grafting methods based on covalent coupling to aminosilane- and epoxysilane-functionalized surfaces were applied to obtain thin CMD layers. The carboxyl moiety of the CMD was coupled to the aminated surface by EDC-NHS reagents, while CMD coupling through epoxysilane molecules was performed without any additional reagents. The surface analysis following the grafting procedures consisted of x-ray photoelectron spectroscopy (XPS), attenuated total reflection infrared spectroscopy (ATR-IR), as well as atomic force microscopy (AFM), which proved the presence and the 1 - 2 nm thickness of the CMD layer, and verified its covalent grafting to the surface. The *in situ* optical waveguide lightmode spectroscopy (OWLS) measurements were suitable to devise the structure of the interfacial dextran layers by the evaluation of the optogeometrical parameters. We found that the extent of the layer anisotropy was dependent both of the grafting procedure (using epoxysilane or aminosylane coatings) and the pH of the CMD solution applied for the grafting (Fig. 1 and 2). The apparent refractive index of the CMD layer was unrealistically higher than the reference values in the literature, which suggested parallel chain conformation to the surface. The developed methodologies allowed to design and fabricate nanometer scale ultrathin CMD layers with well-controlled surface structure, which are otherwise very difficult to characterize in aqueous environments using present instrumentations [1].

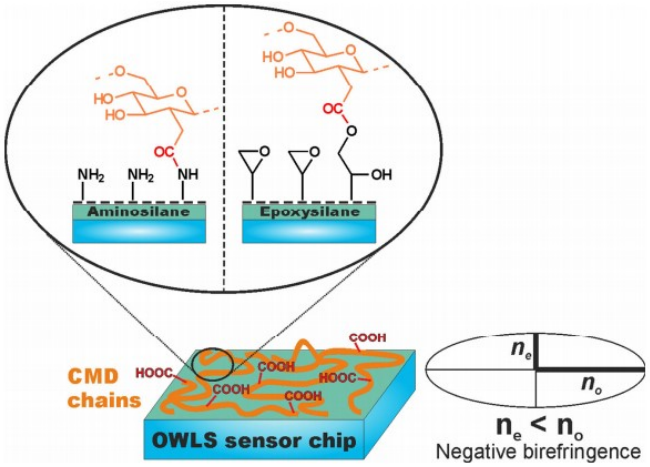

Figure 1 *Schematic illustrations of the CMD grafting chemistries and the supposed structure of the CMD chains on the OWLS sensor chip. The negative birefringence of the layer suggested lain down chains on the surface.*

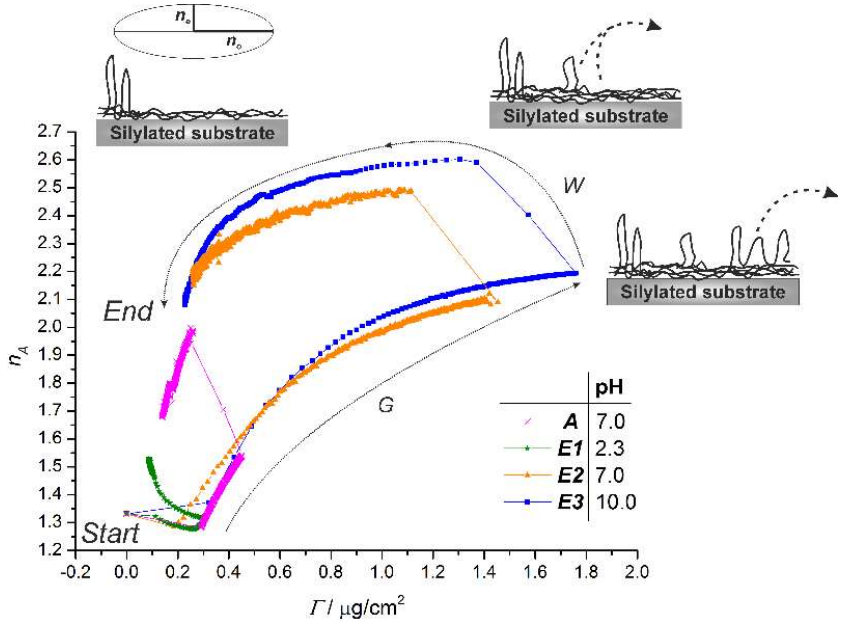

Figure 2 *In situ OWLS measurements on the covalent grafting experiments of the CMD (n_A: apparent refractive index of the CMD layer, Γ: areal mass density of the deposited CMD; A, E: surface with aminosilane or epoxysilane coating, respectively; G, W: grafting or washing sections of the experiment).*

[1] A. Saftics, S. Kurunczi, Z. Szekrényes, K. Kamarás, N.Q. Khánh, A. Sulyok, S. Bősze, R. Horvath. Fabrication and characterization of ultrathin dextran layers: time dependent nanostructure in aqueous environments revealed by OWLS, *Colloids Surfaces B Biointerfaces 146*, 861–870 (2016)

Immune cell adhesion measurements with label-free optical biosensors

Monocytes, dendritic cells (DCs), and macrophages (MFs) are closely related immune cells that differ in their main functions [1]. To study the inherently and essentially dynamic aspects of these cells, dynamic cell adhesion assays were performed with a high-throughput label-free optical biosensor [Epic BenchTop (BT)] on surfaces coated with either fibrinogen (Fgn) or the biomimetic copolymer PLL-g-PEG-RGD [2]. We found that, all three cell types induced a larger biosensor signal on Fgn than on PLL-g-PEG-RGD. The results obtained with evanescent-field-based label-free optical biosensor were compared with three different techniques in this study: the classical fluorescence reader-based adherence assay, the flow chamber technique, and the automated micropipette [3]. The results obtained with the different techniques demonstrate that there are significant differences between the adhesion of the three cell types on Fgn. Monocytes were found to be the less adhesive than MFs and DCs. Furthermore, DCs adhered stronger than MFs; this is in accordance with the fact that DCs have larger expression levels of β2 integrins than MFs (Fig.1). Hence, it seems that the expression levels of β2 integrins fundamentally influence the adhesion capacity of these immune cells. These techniques confirmed the results obtained with the high-temporal-resolution Epic BT, but could only provide end-point data. In contrast, complex, non-monotonic cell adhesion kinetics measured by the high-throughput optical biosensor is expected to open a window on the hidden background of the immune cell–extracellular matrix interactions.

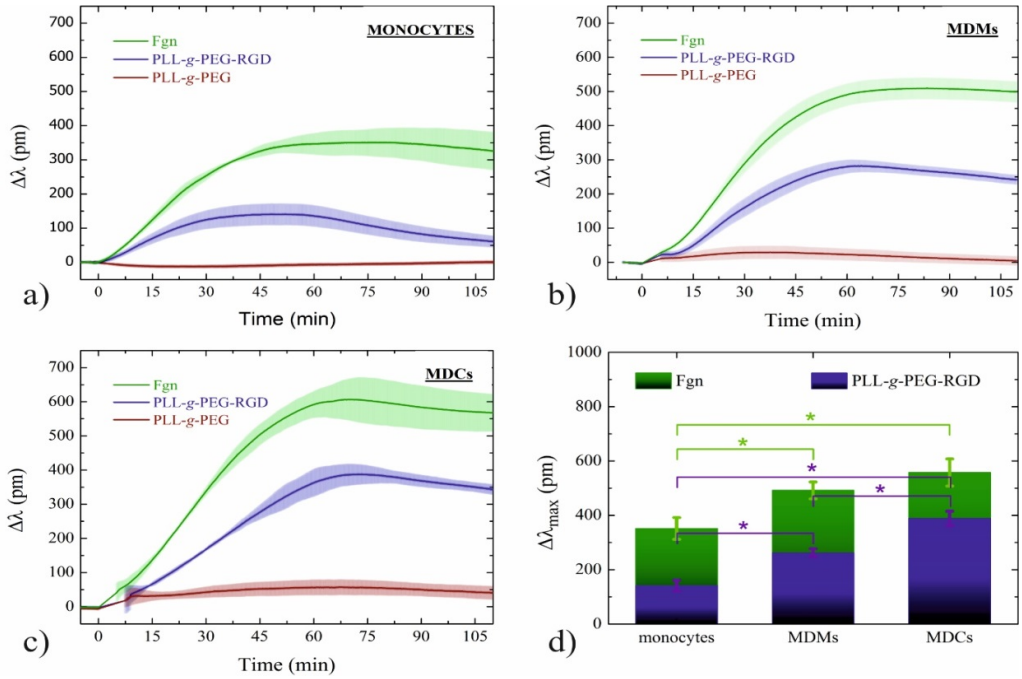

Figure 1 *Time-dependent adherence of monocytes, monocyte-derived macrophages (MFs), and monocyte-derived dendritic cells (DCs) on PLL-g-PEG- and Fgn-coated surfaces, as was measured with the Epic BT in dynamic cell adhesion (DCA) assays.* Representative kinetic profiles provoked by adhering and spreading monocytes, MFs, and DCs are shown in panels a), b), and c), respectively. *PLL-g-PEG-coated surfaces were used as negative control. The background-corrected maximum biosensor signals induced by different cells on PLL-g-PEG-RGD- and Fgn-coated surfaces are shown*

*in panel d). In all panels data are shown as means, error bars represent standard deviations. * indicates statistical significance with p<0.05 (t-test).*

[1] N. Sándor, Sz. Lukácsi, R. Ungai-Salánki, N. Orgován, B. Szabó, R. Horváth, A. Erdei, Zs. Bajtay. CD11c/CD18 Dominates Adhesion of Human Monocytes, Macrophages and Dendritic Cells over CD11b/CD18. *PLoS ONE* 11(9): e0163120. doi:10.1371/journal.pone.0163120 (2016).

[2] N. Orgovan, R. Ungai-Salánki, Sz. Lukácsi, N. Sándor, Zs. Bajtay, A. Erdei, B. Szabó, R. Horvath Adhesion kinetics of human primary monocytes, dendritic cells, and macrophages: Dynamic cell adhesion measurements with a label-free optical biosensor and their comparison with end-point assays. *Biointerphases* 11(3). (2016)

[3] P.K. Jani, E. Schwaner, E. Kajdácsi, M. L. Debreczeni, R. Ungai-Salánki, J. Dobó, Z. Doleschall, J. Rigó Jr., M. Geiszt, B. Szabó, P. Gál, L. Cervenak. Complement MASP-1 enhances adhesion between endothelial cells and neutrophils by up-regulating E-selectin expression. *Molecular Immunology* 75, 38–47. (2016)

CD11c/CD18 Dominates Adhesion of Human Monocytes, Macrophages and Dendritic Cells over CD11b/CD18

Complement receptors CD11b/CD18 (CR3) and CD11c/CD18 (CR4) belong to the family of β2 integrins and are expressed mainly by myeloid cell types in humans. In our study, we analyzed how CD11b and CD11c participate in cell adhesion to fibrinogen, a common ligand of CR3 and CR4, employing human monocytes, monocyte-derived macrophages (MFs) and monocyte-derived dendritic cells (DCs) highly expressing CD11b as well as CD11c [1]. It was determined the exact numbers of CD11b and CD11c on these cell types by a bead-based technique. Applying state-of-the-art biophysical techniques (classical method, computer-controlled micropipette (Fig.1), optical waveguide biosensor [2]), we proved that cellular adherence to fibrinogen is dominated by CD11c. Furthermore, we found that blocking CD11b significantly enhances the attachment of DCs and MFs to fibrinogen, demonstrating a competition between CD11b and CD11c for this ligand. On the basis of the cell surface receptor numbers and the measured adhesion strength we set up a model, which explains the different behavior of the three cell types.

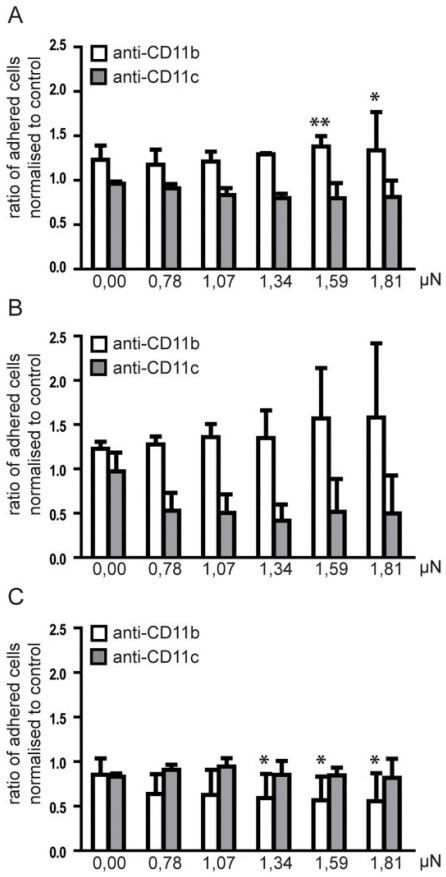

Figure 1 *Force of cell adhesion after blocking CD11b or CD11c with antibodies. The number of adhered cells was determined in the field of the microscope and is shown as 0,00µN. The computer controlled micropipette made serial pick-up processes in the field by using increasing amount of vacuum. The ratio of adhered cells was determined at each lifting force value by dividing the number of adhered cells in the anti-CD11b or anti-CD11c blocked samples by the number of cells in corresponding control sample. Data presented are mean +/-SD of three independent donors samples. Repeated measures ANOVA with Bonferroni post-test was used to determine significant differences compared to control at each force. * = p<0,05, ** = p<0,01.*

In our further experiment we could measure medically relevant adhesion forces between single cells from different cell types [3].

[1] N. Sándor, Sz. Lukácsi, R. Ungai-Salánki, N. Orgován, B. Szabó, R. Horváth, A. Erdei, Zs. Bajtay. CD11c/CD18 Dominates Adhesion of Human Monocytes, Macrophages and Dendritic Cells over CD11b/CD18. PLoS ONE 11(9): e0163120. doi:10.1371/journal.pone.0163120 (2016)

[2] N. Orgovan, R. Ungai-Salánki, Sz. Lukácsi, N. Sándor, Zs. Bajtay, A. Erdei, B. Szabó, R. Horvath Adhesion kinetics of human primary monocytes, dendritic cells, and macrophages: Dynamic cell adhesion measurements with a label-free optical biosensor and their comparison with end-point assays. *Biointerphases* 11(3). (2016)

[3] P.K. Jani, E. Schwaner, E. Kajdácsi, M.L. Debreczeni, R. Ungai-Salánki, J. Dobó, Z. Doleschall, J. Rigó Jr., M. Geiszt, B. Szabó, P. Gál, L. Cervenak Complement MASP-1 enhances adhesion between endothelial cells and neutrophils by up-regulating E-selectin expression. *Molecular Immunology* 75, 38–47. (2016)

Flagellin based biomimetic coatings: from cell-repellent surfaces to highly adhesive coatings

Biomimetic coatings with cell-adhesion-regulating functionalities are intensively researched today. For example, cell-based biosensing for drug development, biomedical implants, and tissue engineering require that the surface adhesion of living cells is well controlled. Recently, we have shown that the bacterial flagellar protein, flagellin, adsorbs through its terminal segments to hydrophobic surfaces, forming an oriented monolayer and exposing its variable D3 domain to the solution [1]. Here, we hypothesized that this nanostructured layer is highly cell-repellent since it mimics the surface of the flagellar filaments. Moreover, we proposed flagellin as a carrier molecule to display the cell-adhesive RGD (Arg-Gly-Asp) peptide sequence and induce cell adhesion on the coated surface. The proposed concept is visualized in Fig. 1.

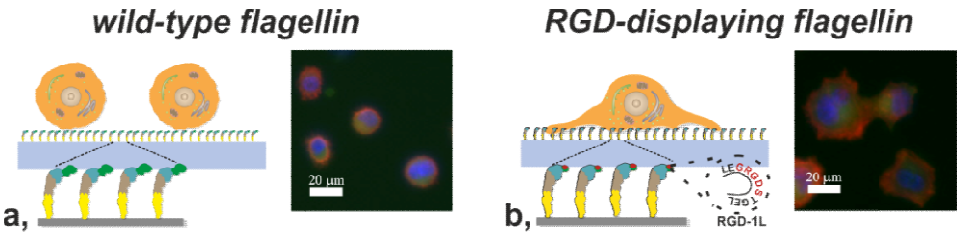

Figure 1 *Schematic representation and fluorescent microscopic images of cells on anti-adhesive and adhesive surface coatings. a, Wild type flagellin hinder cell adhesion on the sensor surface. b, RGD displaying flagellin induce cell adhesion and spreading.*

The details of this work have been recently published in Acta Biomaterialia [2] and summarized as follows. During the mammalian HeLa cell adhesion experiments, we recorded the cell adhesion data with OWLS (Optical Waveguide Lightmode Spectroscopy) and applied a PLL-*g*-PEG polymer monolayer as reference anti-adhesive surface coating. The ΔN_{TM} (effective refractive index change) signals recorded on surfaces coated with either wild-type flagellin or PLL-*g*-PEG both remained at the level of the baseline during the whole time span of the cell-based assay (Fig. 2a). Since ΔN_{TM} is proportional to the degree of the cell adhesion [3], we concluded that both surfaces were highly cell-repellent. The changes in the full width of the TM_0 resonant peak at half maxima (changes of *W, i.e.* ΔW) were also monitored [4]. Cellular activity provoked negligible shifts in *W,* further proving that there was no significant cell adhesion on either wild-type flagellin or PLL-*g*-PEG layers (Fig. 2b). Cell adhesion and spreading would make ΔW increase due to the micron-scale optical inhomogeneities caused by the adhering cells at the sensor surface. Our conclusions are well supported by the phase contrast images of cells captured on the coated sensor surfaces after the OWLS measurements (Fig.3).

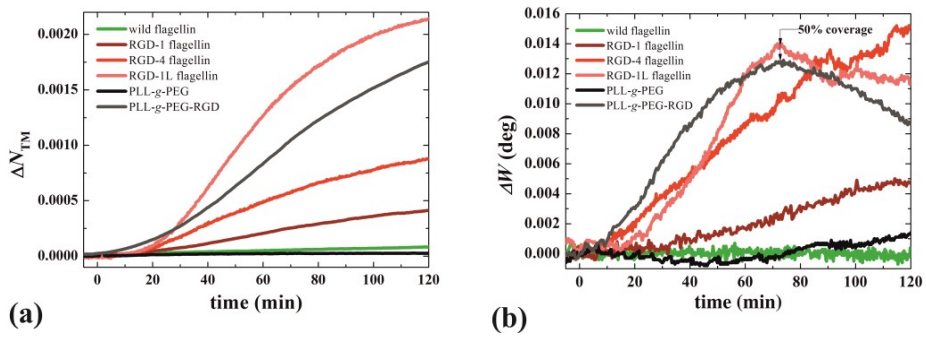

Figure 2 OWLS signals recorded after living cells were seeded on the surfaces of the OWLS chips coated with the indicated layers. (a) The measured ΔN_{TM} values correlating with the strength of cellular adhesion. (b) Temporal evolution of the width of the OWLS resonance peak (W).

We created flagellin-based fusion proteins in which the D3 domain of FliC is replaced by an oligopeptide segment containing one or more of the integrin binding RGD motif. Although the simple RGD motif is sufficient to mediate binding to integrin receptors, the flanking amino acid residues may further enhance binding affinity. Thus, we chose the more efficient GRGDS pentapeptide as the basic unit for insertion.

We tested the genetically modified flagellin variants as surface coatings, too. On the sensor surfaces coated with RGD-displaying flagellin variants, cells provoked a continuous increase (up to a maximum value) in N_{TM}. Cells seeded on the PLL-*g*-PEG-RGD-coated adhesive reference surface produced similar signals. The observed saturating sigmoid-shaped curves are clear characteristics of active cell spreading [5].The larger the signal the stronger the cellular adhesion; cells induced the largest ΔN_{TM} signal thus showed the strongest adherence on the RGD-1L surface. The second, the third and the fourth highest cell adhesion were obtained with the PLL-*g*-PEG-RGD, RGD-4 and RGD-1 coatings, respectively (see Fig. 2a).

In conclusion, cells on the three different RGD-displaying flagellin layers adhered to different degrees, which could be explained by the fact that the RGD motif was integrated with different linker pairs, and most probably provided different accessibility and flexibility for the cells.

The temporal evolution of the width of the OWLS resonance peak (*W)* during cell adhesion was also recorded (Fig. 2b). In the case of the RGD-1 and RGD-4 flagellin-coated surfaces *W* increased monotonically and continuously until the end of the experiment. This means that the surface coverage did not reach 50% during the time of the experiment. In contrast, in case of the PLL-*g*-PEG-RGD and RGD-1L surfaces ΔW first increased, reached a very clear maximum, and then decreased. Previous experiments and numerical simulations suggest that such kinetic behavior indicates that the cell coverage increased above 50%. The half surface coverage is reached at the maximum value of the resonant peak width [4]. This finding also suggests the most intense cell adhesion and spreading took place on the PLL-*g*-PEG-RGD and RGD-1L coatings. The phase contrast images recorded well confirm the above findings (see Fig.3).

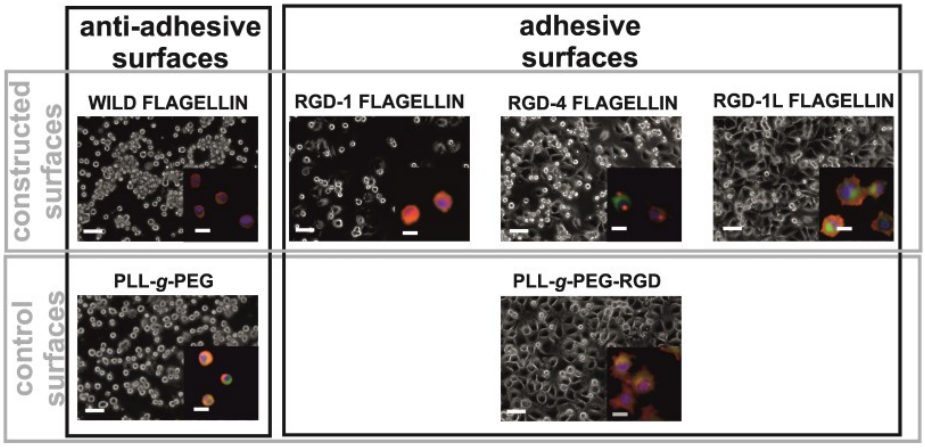

Figure 3 Microscope images of the cells seeded on the investigated surfaces. The phase contrast images (scale bar 50 µm) show cells on the biosensor surfaces right after the OWLS experiments. The insets (scale bar 20 µm) show fluorescently labeled cells cultured on glass coverslips in parallel experiments (red: F-actin, blue: nuclei, green: vinculin).

In conclusions, the application of flagellin-based fusion proteins for fabricating tunable surfaces opens up new avenues. Wild-type flagellin and its functionalized variants can be applied together in various mixing ratios. Thus, oriented affinity layers with negligible non-specific cell adhesion can be created with well controlled average distance between the binding sites. Other peptide sequences can be easily introduced into flagellin in a similar manner. Therefore, surfaces with multiple functionalities can be fabricated in a straightforward manner. Flagellin variants exhibiting specific recognition functionalities offer the possibility to create a large variety of coatings on hydrophobic surfaces. Since flagellin does not adsorb at all on hydrophilic surfaces solution [1], by patterning the hydrophobicity of the surfaces and exposing them to the solutions of functionalized flagellin, patterned functional surfaces could be easily created in a straightforward and cost-effective manner.

[1] N. Kovacs, D. Patko, N. Orgovan, S. Kurunczi, J. J. Ramsden, F. Vonderviszt, and R. Horvath, "Optical anisotropy of flagellin layers: in situ and label-free measurement of adsorbed protein orientation using OWLS," *Anal. Chem.* 85, 11, 5382–9, (2013)

[2] B. Kovacs, D. Patko, I. Szekacs, N. Orgovan, S. Kurunczi, A. Sulyok, N. Q. Khanh, B. Toth, F. Vonderviszt, and R. Horvath, "Flagellin based biomimetic coatings: from cell-repellent surfaces to highly adhesive coatings," *Acta Biomater.* 42, 66–76, (2016)

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Label-free optical biosensor for real-time monitoring the cytotoxicity of xenobiotics: a proof of principle study on glyphosate

Rapid and inexpensive biosensor technologies allowing real-time analysis of biomolecular and cellular events have become the basis of next-generation cell-based screening techniques, capable to indicate organic and inorganic environmental pollutants including genotoxic, ecotoxic or endocrine disrupting xenobiotic compounds. Our work opens up novel opportunities in the application of high-throughput label-free optical biosensor Epic BenchTop (BT) in cell toxicity studies. Effects of xenobiotics can lead to passive cell death or result in the active mechanism of apoptosis. These integrated cellular events can be detected through differences in the kinetics of processes induced by the given xenobiotic in the 150 nm layer above the sensor surface. Epic BT was successfully applied to identify the effect of a formulated herbicide Roundup Classic and its components (active ingredient glyphosate and co-formulant polyethoxylated tallow amine (POEA)) on MC3T3-E1 preosteoblast cells adhered on the sensor surface. Moreover, we subsequently validate the data obtained from the Epic assays with microscopic and flow cytometric techniques.

The half maximal inhibitory concentration of Roundup Classic, POEA and glyphosate upon 1 h of exposure was found to be 0.024%, 0.021% and 0.163% in serum-containing medium and 0.028%, 0.019% and 0.538% in serum-free conditions, respectively (at concentrations equivalent to the diluted Roundup solution) (Fig. 1). These results showed a good correlation with parallel end-point assays, demonstrating the outstanding utility of the Epic technique in cytotoxicity screening, allowing not only high-throughput, real-time detection, but also reduced assay run time and cytotoxicity assessment at end-points far before cell death would occur.

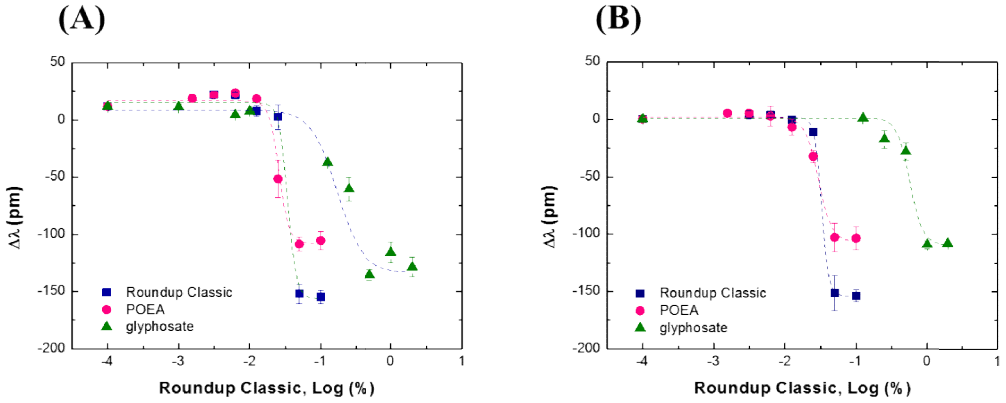

Figure 1 *Dose response curves on MC3T3-E1 cells in α-MEM+10% FBS (A) or in 20 mM HEPES HBSS buffer (B). Data represented are mean ± SD of identical experiments made in three replicates.*

Self-assembled, nanostructured coatings for water oxidation by alternating deposition of Cu-branched peptide electrocatalysts and polyelectrolytes

In the past few decades the importance of the LbL (Layer-by-Layer) technique has grown, especially to build up functional multilayers or surface-based nanodevices and surface modifications. The LbL method is based on the alternating exposure of a surface to oppositely charged polyelectrolytes. Those polymers are called polyelectrolytes (PEs), whose repeating units are ionic groups and have high molecular weight. The PLL (poly-L-lysine) and PAH (poly-(allylamine hydrochloride)) are positively charged PEs, these were used in our experiments. The advantages of the PEs have been taken to build heterogeneous catalytic layers with Cu-peptides on indium-tin-oxide (ITO) conducting OWLS chip surface, with the use of the principle of the LbL technique. The Cu-peptides were known to accelerate the half-reaction of water oxidation that has significance in envisioned water splitting systems to generate H2 as renewable fuel. The peptide ligands bind to the metal in the equatorial positions at high pH and capable of stabilizing higher oxidation states occurring in catalysis. The applied complexes contain L-2,3-diaminopropionic acid (dap) linkers that provide branched structure with a glycine (Cu-3G) or a histidine (Cu-2GH) residue at the C-terminus.

Initially suitable polyelectrolyte pairs were sought to the Cu-peptides and Optical Waveguide Lightmode Spectroscopy (OWLS) was applied to monitor the build-up process of the nanocomposite multilayers in situ. The Cu-2GH paired with PAH and Cu-3G coupled with PLL were suitable for further studies. The layer-buildup- mechanism is supposed to involve secondary binding forces beside the electrostatic interactions. Phosphate is also incorporated into the layers.

Optimization was also supported by electrochemical methods. Conditions of pH 9-10 and Cu-complex concentration of 0.1-0.5 mM were optimal for stable LbL formation. These layers show electrocatalytic activity at high positive (>1.1 V) potential and the measurement on EC-OWLS (OWLS with electrochemical function) shows, that the surface mass density is somewhat decreased after the alternating potential cycles, but the layers don't lose their activity (Fig.1).

Other surface analyses included XPS (X-ray photoelectron spectroscopy) to detect the Cu(II) in an organic N-donor environment, and AFM that shows an inhomogeneous surface with nanoporous structure. The profilometric section demonstrates that the Cu-2GH multilayers are smoother and thinner, than that of Cu-3G.

In conclusion, with the use of OWLS/EC-OWLS it was possible to examine functional thin layers and define their properties. The Cu-peptide/PE film is electrochemically active stable, heterogeneous catalyst layers. This catalyst heterogenization method could lead to a cheaper, more economic and environmentally friendly technology [1].

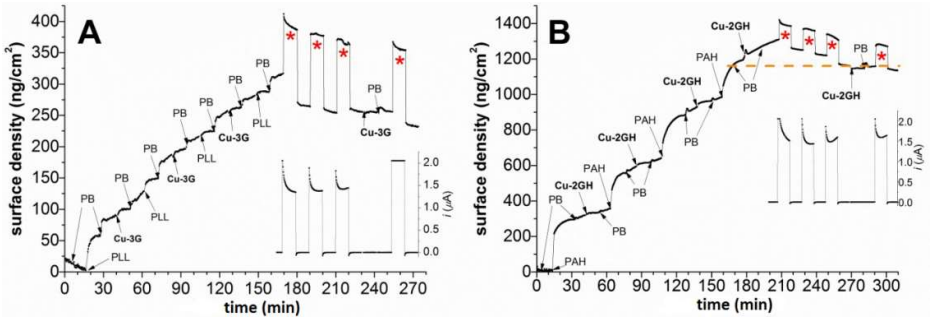

Figure 1 *(A) three deposition cycles of PLL and Cu-3G (0.5 mM) in phosphate buffer (PB) (0.1 M) at pH = 10.12 and concomitant 3×10 min runs of CPE at 1.1 V vs. Ag/AgCl with intermittent breaks of 10 minutes and concomitant attempt to supply the electrolyzed surface with Cu-3G at an ITO-coated chip placed in an EC-OWLS cell; (B) the same sequence applied to PAH and Cu-2GH layers.*

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Self-assembly and structure of flagellin–polyelectrolyte composite layers: polyelectrolyte induced flagellar filament formation during the alternating deposition process

The simple and cost-effective bottom-up fabrication of complex functionalized nanostructures is extensively researched today. Here, the alternating deposition of the negatively charged protein flagellin and a positively charged polyelectrolyte are studied. The multilayer buildup was followed in situ using Optical Waveguide Lightmode Spectroscopy (OWLS) revealing the deposited surface mass density in real time during the alternating deposition process. The nanostructure of the assembled films was investigated by Atomic Force Microscopy (AFM) measurements. When flagellin was applied in its natural filamentous form no distinct multilayer buildup was observed, the filaments assembled mainly into bundles. In contrast, when thermally treated filament solution or pure flagellin monomer solution was used a systematic linearly growing buildup was seen, and thick, relatively smooth films were fabricated. The structural investigation (Fig.1) revealed that the fabricated films are relatively smooth, what is in close connection with the assembling mechanism, having a tendency of filling up possible holes. We also concluded that the flagellin monomers assembled into nanofilaments inside the multilayer and even a single layer of polycation could induce the self-assembly of filaments, possibly by helping the adsorption of flagellin monomers close to each other. Both the filament formation and the multilayer buildup were completely absent when a truncated flagellin variant – missing the disordered terminal regions – was applied. Since these regions are necessary for filament formation, we conclude that the linearly growing nature of the layer is a clear consequence of filament formation.

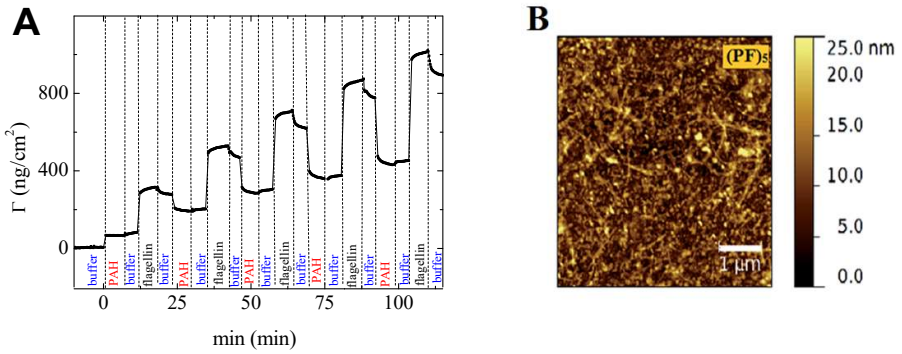

Figure 1 *shows A, Formation of 5 bilayer of flagellin and PAH polyelectrolyte monitored by OWLS. B, AFM picture of 5 bilayer of flagellin and PAH polyelectrolyte*

Therefore, this study first reveals a new type of linearly growing polyelectrolyte multilayer buildup mechanism, when one of the components induces the self-assembly of the oppositely charged component, creating a complex, stable and smooth filamentous nanostructured coating. These composite films can find diverse applications in nanotechnology and in biomedical sciences since the variable D3 domain of flagellin subunits can be easily modified to express enzymatic, fluorescent or molecular binding properties on the surfaces of the filaments. The present work therefore opens up novel routes in the bottom-up fabrication of complex nanostructured coatings [1].

[1] E. Farkas, D. Patko, N.Q. Khanh, E. Toth, F. Vonderviszt, R. Horvath. Self-assembly and structure of flagellin–polyelectrolyte composite layers: polyelectrolyte induced flagellar filament formation during the alternating deposition process. *RSC Adv.*, 6, 92159-92167 (2016)

Label-free optical biosensor for real-time monitoring

of single cell adhesion and signaling

Recently, the research and development exploring interactions at individual cellular levels are emerging. One of the reasons is that often the cell populations from the same source do not form a genetically homogeneous set, so in many clinical applications, such as targeted therapies, problems may arise due to the inhomogeneity of the cell population. Due to the recent technological advances in real-time, label-free optical biosensors, it has become possible to produce large lateral resolution devices that can be used to measure live cell adhesion and signaling in individual cells. The topic of our work was to investigate the adherence of unique cancerous mammalian cells, and their interaction with certain biologically active molecule-induced stimulators by using qualitative and quantitative image processing techniques. The results can answer whether the cellular responses of individual cells are consistent with the biosensor signal obtained from the average response of thousands of cells. Moreover, the differences between the cells from the different tissues will be quantifiable according to the parameters studied.

Our work also looks for the parameters that can be used to isolate smaller subpopulations from a particular cell population. In the first part of our research, calibration measurements were performed on the Epic BT Cardio System, investigating the response of the biosensor due the change of the refractive index in the examined medium. The second part of our research included multiple measurements on single human immortalized cancer cell lines (HeLa and BJAB). The measurements investigated the adhesion and signaling of the single cells adhered on different coatings. The cell signaling events were induced using B-cell receptor specific antibodys for BJAB cells, and histamine solutions on the HeLa cells. Data evaluation techniques included fitting simple logistic equation to the adhesion part of the biosensor signal to acquire the parameters associated with the adhesion event, and recording the difference between the biosensor response signal of the control, and induced cell lines in the signaling part of the measurements. The fitted parameters were compared to each other for the different cell lines and coatings.

Our results indicates that it is possible using this technique to differentiate the different cell line types on different coatings based on only the fitted parameters of the adhesion part of the measurements (Fig. 1-5).

We also acquired significant results between the induced, and control cell lines in respect of the signaling part of the experiments.

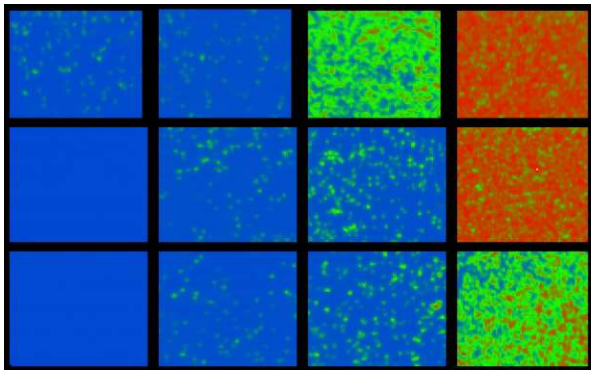

Figure 1 *The acquired biosensor signal distribution on the the biosensor wells.*

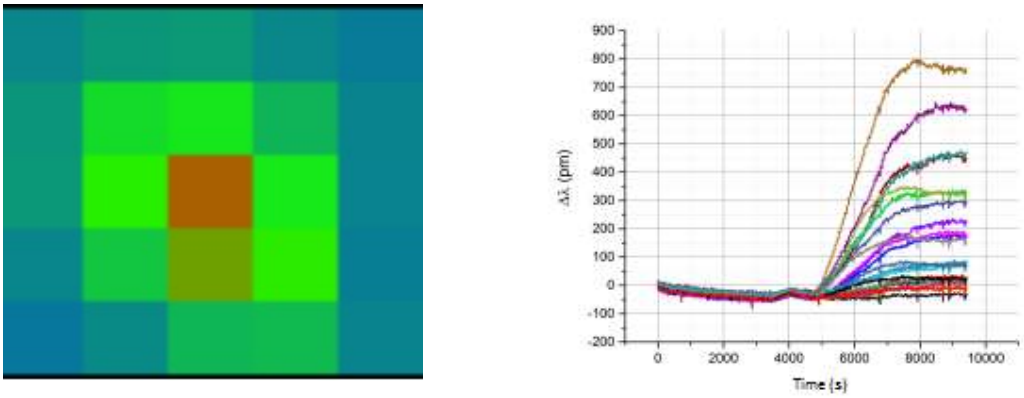

Figure 2 *Left: The biosensor signal distribution for a single HeLa cell. Right: The time series for the adhesion event for a single HeLa cell. The individual curves represents different 21·21 µm areas on the biosensor chip surface associated with the cell.*

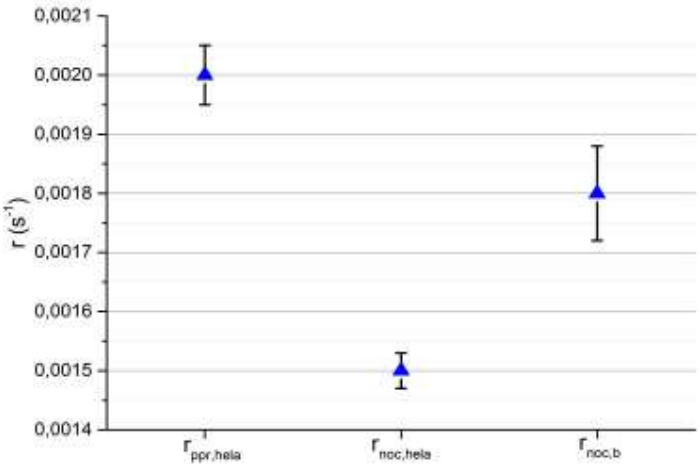

Figure 3 *The comparison of the adhesion parameter (spreading rate constant) of different cell lines on different coatings.*

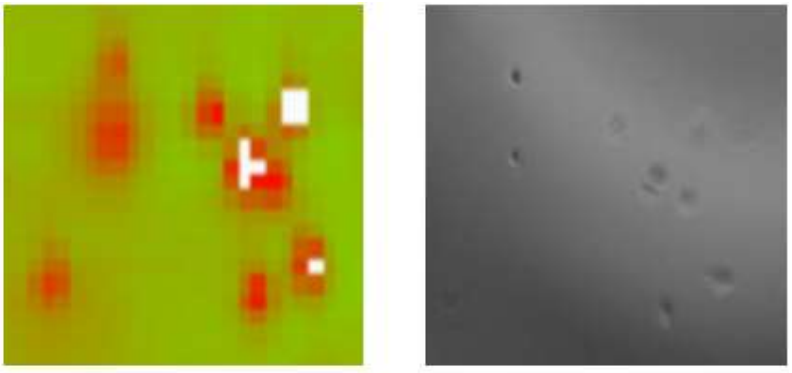

Figure 1 *The comparision between the biosensor signal distribution (left) and the microscope image (acquired with a phase contrast microscope, right) of the cells attached to the surface of the sensor.*

Figure 5 *The comparision between the biosensor signal distribution and the real image (acquired with a phase contrast microscope, right) of the cells attached to the surface of the sensor.The biosensor signal increases well correlate with the positions of individual cells.*

Nanourf Flex AFM installation

Nanosurf Flex AFM has been installed in our laboratory along with the FluidFM system from an MTA Instrumental fund. The AFM is capable for cell imaging beyond the conventional surface topography which gives an invaluable help in our work. The AFM system can be run in force spectroscopy mode which we will use for the characterization of thin coatings and protein layers developed in our laboratory (Fig.1).

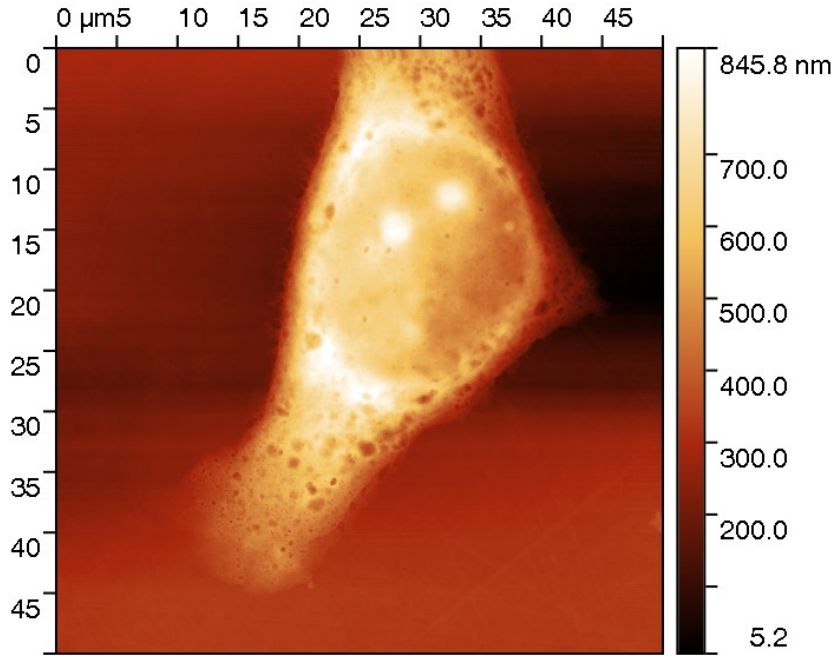

Figure 1 *AFM image of a fixed HeLa cell from the Nanosensorics laboratory, 2017*

The chemical patterning capability of FluidFM is an important line of our research. CMD (carboxymethyl dextran) coatings were tested for their cell-repellent nature. Our results were obtained for CMD surface patterns prepared by our novel deposition method based on the Fluid FM system. The printed patterns on the surface with cyclic RGDfk peptide motifs were tested for cell adhesion on HeLa cells (see Fig.2).

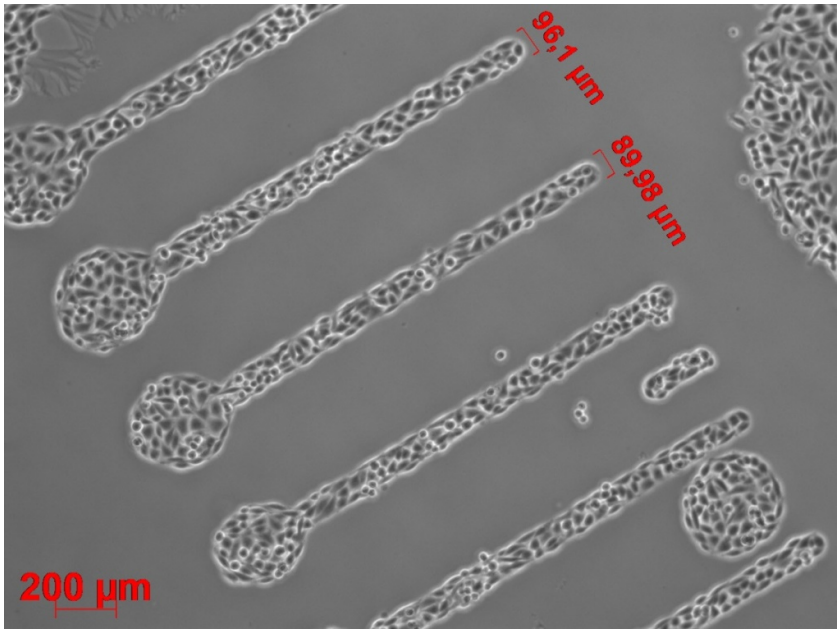

Figure 2 *Adhesion of HeLa cells onto printed patterns on the surface with cyclic RGDfk peptide motifs.*

Novel biophysical methods for measuring single cell adhesion forces

Our goal was to test and compare two new methods that can measure adhesion forces at the single cell level. The first device was a high throughput computer controlled micropipette which is capable of measuring adhesion forces on 100+ cells in a 30 minute time. Its working principle is the step pressure method which consists of sequential applying of underpressure on the cells. The suction forces are calculated by computer simulations of the system. The output of a measurement is a distribution of adherent cells in respect of applied suction forces, from which it is possible to define a mean adhesion force characteristic to the cell population (see Fig. 1).

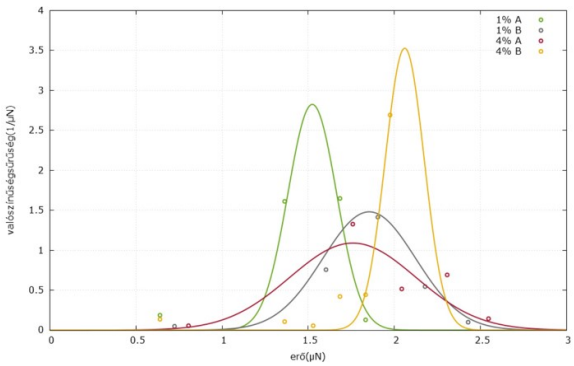

Figure 1: *PDFs of adhesion forces from micropipette measurements on 4 different coatings*

The second device was the FluidFM BOT, a special robotic AFM equipped with microfluidics, which can measure the direct forces acting between a cell and the surface. This method has a lower throughput in return for the higher sensitivity, which is apparent from the measured adhesion curve of a single cell, showing characteristic cell detachment events (Figure 2). The first part of the curve shows the approaching of the AFM tip to the cell, and the red part taking place in the attractive, negative force region shows the adhesion of the cell. The highest absolute force in the adhesion region defines the adhesion force of a cell, and the integral of the curve is the work required to detach the cell from the surface.

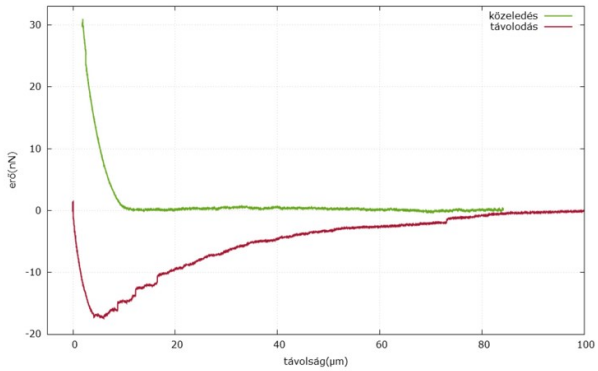

Figure 2: *A typical single cancer cell adhesion curve from a FluidFM BOTmeasurement*

For the comparison of the two techniques identical PLL-PEG/PLL-PEG-RGD surface coatings were applied prior the incubation of HeLa cells. The results of the measurements are shown in Figure 1 for the micropipette technique and in Figure 3 for the FluidFM BOT. The results showed a 40-fold difference in adhesion forces between the two techniques, which actuates further investigations. Our next step in the comparison is to develop a manageable model system with fewer variables and easier geometries.

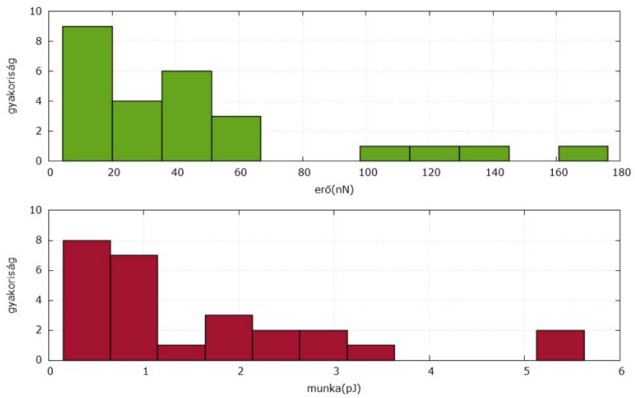

Figure 3: *Distributions of adhesion forces (green) and adhesion work (red).*