Expanding the frontiers of label-free single cell biosensing and manipulation in biomedical engineering
FINAL REPORT

WP1 -Label-free instrumentation and methodology development

The main objectives were to develop highly sensitive and reliable label-free high-content optical methods, capable of monitoring single cells from a heterogeneous population of cells. The optical sensors will be combined with single-cell manipulation techniques (micropipette, Fluid FM BOT) to pick up and position the individual cells to be studied. Advanced fluid handling solutions will be also developed to control cell deposition and the flow velocity profile over the employed cells and tissue models. The main objectives were to develop highly sensitive and reliable label-free high-content optical methods. We have focused on the combination of FluidFM, computer-controlled micropipette, and optical biosensors (Resonant Waveguide Grating (RWG) and Grating Coupled Interferometry (GCI)) for innovative measurements.

Results linked to the robotic FluidFM:

FluidFM technique can be considered as the nanofluidic extension of the atomic force microscope (AFM). This novel instrument facilitates the experimental procedure and data acquisition of force spectroscopy (FS) and is also used for the determination of single-cell adhesion forces (SCFS) and elasticity. FluidFM uses special probes with an integrated nanochannel inside the cantilevers supported by parallel rows of pillars. However, little is known about how the properties of these hollow cantilevers affect the most important parameters that directly scale the obtained spectroscopic data: the inverse optical lever sensitivity (InvOLS) and the spring constant (k). The precise determination of these parameters during calibration is essential to gain reliable, comparable, and consistent results with SCFS. As demonstrated by our literature survey, the standard error of previously published SCFS results obtained with FluidFM ranges from 11.8% to 50%. The question arises whether this can be accounted for biological diversity or may be the consequence of improper calibration. Thus the aim of our work was to investigate the calibration accuracy of these parameters and their dependence on (1) the aperture size (2, 4, and 8 μm) of the hollow micropipette type cantilever; (2) the position of the laser spot on the back of the cantilever; (3) the substrate used for calibration (silicon or polystyrene). It was found that both the obtained InvOLS and spring constant values depend significantly on the position of the laser spot. Based on our results a calibration strategy is proposed and the optimal laser position which yields the most reliable spring constant values was determined and found to be on the first pair of pillars (*Nagy et al. Scientific Reports, 2019*).

In our other work, high spatial and temporal resolution resonant waveguide grating based label-free optical biosensor was combined with robotic fluidic force microscopy to monitor the adhesion of single living cancer cells. In contrast to traditional fluidic force microscopy methods with a manipulation range in the order of 300–400 micrometers, the robotic device

employed here can address single cells over mm-cm scale areas. This feature significantly increased measurement throughput and opened the way to combine the technology with the employed microplate-based, large-area biosensor. After calibrating the biosensor signals with the direct force measuring technology on 30 individual cells, the kinetic evaluation of the adhesion force and energy of large cell populations was performed for the first time. We concluded that the distribution of the single-cell adhesion force and energy can be fitted by log-normal functions as cells are spreading on the surface and revealed the dynamic changes in these distributions (Figure 1). The present methodology opens the way for the quantitative assessment of the kinetics of single-cell adhesion force and energy with an unprecedented throughput and time resolution, in a completely non-invasive manner (*Sztilkovics et al. Scientific Reports, 2020*).

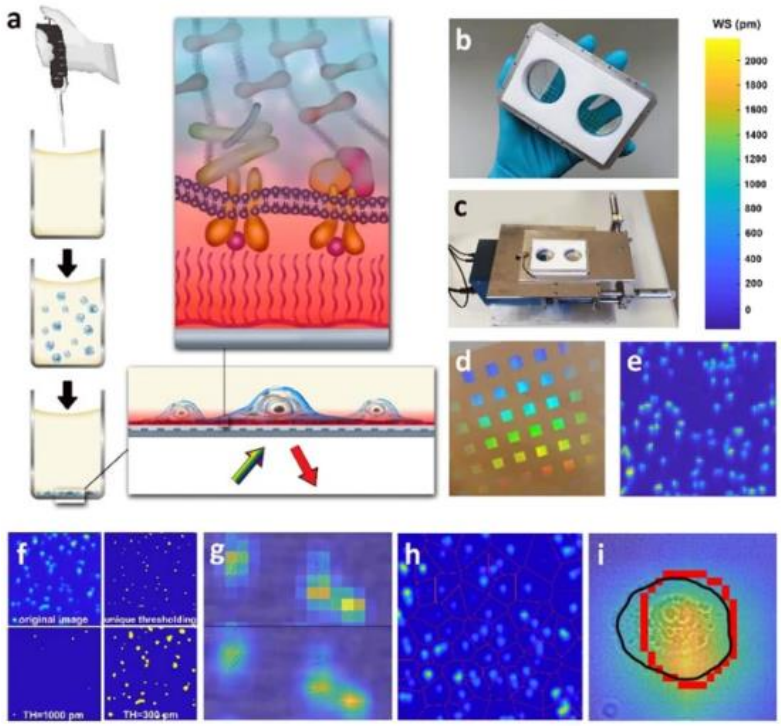

Figure 1*. The optical biosensor measurement workflow and results. (a) Schematic of the measurement workflow. (b,c) Photographs showing the custom-made biosensor insert holder (in a hand, and placed into the Epic Cardio device) with two circular wells optimized for subsequent FluidFM BOT measurements. (d) Photograph of the Epic Cardio biosensor insert. (e) Raw WS signal image of a single sensor area at t = 90 min. (f) Comparison of different thresholding strategies of recorded biosensor images. (g) Fused image of the biosensor signal and the brightfield picture, showing a clear correspondence between the two overlapping modalities. (h) The Voronoi tessellation of a sensor area. (i) Area matching segmentation: the combined optical biosensor and brightfield picture shows how the segmented cell perimeter (red) approximates the actual cell perimeter measured on the microscope image (black) after setting the optimal threshold.*

Computer algorithms were developed to find the same cells on the microscope and biosensor images. This will facilitate effective single-cell isolation based on label-free data. We have also developed a novel assay to monitor cancer cell invasion into compact cell layers using quantitative phase imaging (*Nagy et al. Scientific Reports, 2022*). We continued the combined optical biosensor and FluidFM cell adhesion measurements using various surfaces and cell types.

We studied the cellular adhesion maturation of epithelial Vero monolayers by measuring single-cell force-spectra with FluidFM. We found that HeLa cells adhere significantly stronger to the tight Vero monolayer than cells of the same origin. Moreover, the mechanical characteristics of Vero monolayers upon cancerous HeLa cell influence were recorded and analyzed (*Nagy et al.. European Journal of Cell Biology, 2022*). In our other study, FluidFM was utilized to measure the adhesion parameters of cells in a high-throughput manner to study their population distributions in-depth. The investigated cell type was the genetically engineered HeLa Fucci construct with cell cycle-dependent expression of fluorescent proteins. We first revealed that reticular adhesions can exert a higher force per unit area than canonical focal adhesions, and cells in this phase are significantly stiffer (*Nagy et al. Scientific Reports, 2022*).

In a book chapter, we presented and summarized the fluidic force microscopy and related biosensors for medical applications (*Gerecsei et al. Nanobioanalytical Approaches to Medical Diagnostics. Woodhead Publishing Series in Biomaterials, 2022*). The work on the development of rotating fluid configurations also continued, we significantly miniaturized our relevant biosensor setup.

Results by computer controlled micropipette (solely or combined with FluidFM)

We demonstrated that a computer controlled micropipette (CCMP) is a straightforward and high-throughput alternative to quantify the surface adhesion of functionalized microparticles. However, being an indirect force measurement technique, its in-depth comparison with a direct force measurement is a prerequisite of applications requiring calibrated adhesion force values. To this end, we attached polystyrene microbeads to a solid support by the avidin-biotin linkage. We measured the adhesion strength of the microbeads with both a specialized robotic fluid force microscope (FluidFM BOT) and CCMP (Figure 2). Furthermore, the bead-support contact zone was directly characterized on an optical waveguide biosensor to determine the density of avidin molecules. Distribution of the detachment force recorded on ∼50 individual beads by FluidFM BOT was compared to the adhesion distribution obtained from CCMP measurements on hundreds of individual beads. We found that both methods provide unimodal histograms. We conclude that FluidFM BOT can directly measure the detachment force curve of 50 microbeads in 150 min. CCMP can provide calibrated binding/adhesion force values of 120 microbeads in an hour (*Gerecsei et al. Journal of Colloid and Interface Science, 2019*).

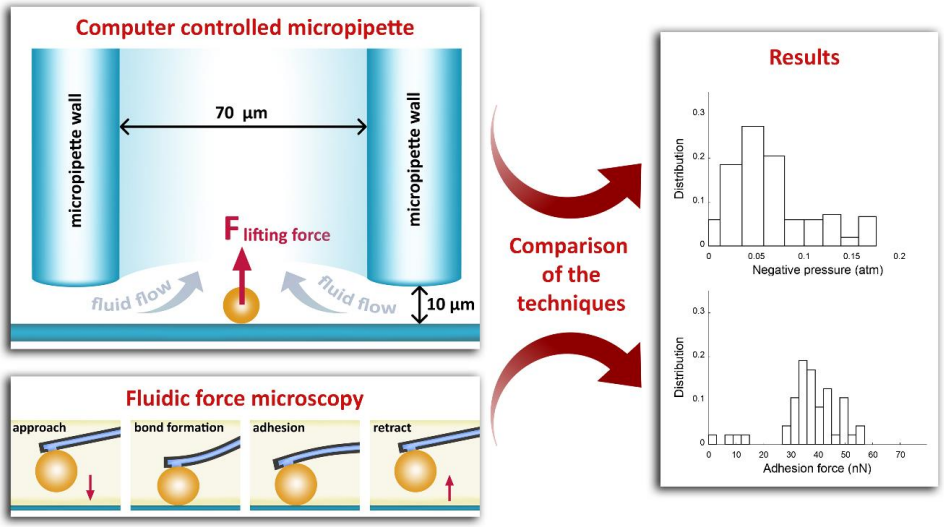

Figure 2. *Schematic illustration of the methods. Adhesion force measurements on microbeads were performed by using computer controlled micropipette (CCMP) and Fluidic force microscopy. The received results of the two techniques has been compared.*

Fluidic force microscopy and optical methods were employed to study cell adhesion on functionalized surfaces. Although microliter-scale liquid handling with a handheld pipette is a routine task, pipetting nanoliter-scale volumes is challanging due to several technical difficulties including surface tension, adhesion and evaporation effects. We developed a fully automated piezoelectric micropipette with a precision of < 1 nanoliter, improving the efficiency of imaging-based single-cell isolation to above 90%. This improvement is crucial when sorting rare or precious cells, especially in medical applications. The compact piezoelectric micropipette can be integrated into various (bio)chemical workflows. It eliminates plastic tubes, valves, syringes, and pressure tanks. For high-quality phase-contrast illumination of the sample, e.g., cells or tiny droplets, we constructed rings of LEDs arranged concentrically to the micropipette. The same device can be readily used for single-cell printing and nanoliter-scale droplet printing of reagents using either fluorescent or transparent illumination on a microscope. We envision that this new technology will shortly become a standard tool for single-cell manipulations in medical diagnostics, e.g., circulating tumor cell isolation (*Francz et al. Microfluidics and Nanofluidics, 2020*).

We applied a computer-controlled micropipette (CCMP) to measure the dissociation constant (Kd) of integrin-RGD-binding. Surface coatings with varying RGD densities were prepared, and the detachment of single cells from these surfaces was measured by applying a local flow inducing hydrodynamic lifting force on the targeted cells in discrete steps (*Gerecsei et al. Biosensors, 2021*).

In the other article (*Ungai-Salánki et al. Journal of Colloid and Interface Science, 202*1), we applied standard computational fluid dynamics simulations to reveal the detailed physical background of the flow generated by the micropipette when probing microbead adhesion on functionalized surfaces. Measuring the aspiration pressure needed to pick up the biotinylated

10 μm beads on avidin coated surfaces and converting it to a hydrodynamic lifting force on the basis of simulations.

We investigated the integrin-mediated adhesion between cancer cells and their RGD (Arg-Gly-Asp) motif displaying biomimetic substratum using the HeLa cell line transfected by the Fucci fluorescent cell cycle reporter construct. We employed a computer-controlled micropipette and a high spatial resolution label-free resonant waveguide grating-based optical sensor calibrated to adhesion force and energy at the single-cell level. We found that the overall adhesion strength of single cancer cells is approximately constant in all phases except the mitotic (M) phase with a significantly lower adhesion (*Ungai-Salánki et al. Scientific Reports, 2021*).

Water in oil emulsions have a wide range of applications, from chemical technology to microfluidics, where the stability of water droplets is of paramount importance. Here, using an accessible and easily reproducible experimental setup, we describe and characterize the dissolution of water in oil, which renders nanoliter-sized droplets unstable, resulting in their shrinkage and disappearance in a time scale of hours. This process has applicability in creating miniature reactors for crystallization or potentially for single living cells. We generated nanoliter-sized water droplets dispersed in oil and observed that they gradually shrank and disappeared in a few hours. Using an easily reproducible and low-cost setup based on a spinning Petri dish to generate the nanoliter scale droplets and an inverted optical microscope to observe them, we monitored the contact radius and the volume of droplets, without the need for specialized droplet printing equipment (*Gerecsei et al. Colloids and Interfaces, 2022*).

Classification of live or fixed cells based on their unlabeled microscopic images would be a powerful tool for cell biology and pathology. For such software, the first step is the generation of a ground truth database that can be used for training and testing AI classification algorithms. The Application of cells expressing fluorescent reporter proteins allows the building of ground truth datasets in a straightforward way. In our other study, we presented an automated imaging pipeline utilizing the Cellpose algorithm for the precise cell segmentation and measurement of fluorescent cellular intensities across multiple channels. We analyzed the cell cycle of HeLa–FUCCI cells expressing fluorescent red and green reporter proteins at various levels depending on the cell cycle state. To build the dataset, 37,000 fixed cells were automatically scanned using a standard motorized microscope, capturing phase contrast and fluorescent red/green images. Our pipeline allows the high-throughput and accurate measurement of cellular fluorescence providing extensive statistical information on thousands of cells with potential applications in developmental and cancer biology. Furthermore, our method can be used to build ground truth datasets automatically for training and testing AI cell classification. Our automated pipeline can be used to analyze thousands of cells within 2 h after putting the sample onto the microscope (*Cheragi et al. Heliyon, 2024*).

Results linked to the resonant waveguide grating (RWG) technique:

Label-free optical biosensors are powerful tools for the real-time monitoring of both molecular and cellular-scale interactions. Resonant waveguide grating biosensors are based on the detection of refractive index changes induced by molecular interactions and/or cell mass redistributions. The Epic BenchTop and Epic Cardio are two biosensors with high

sensitivity and throughput that offer excellent potential for life science research. Both instruments are suitable for cell-based and biochemical assays. In our paper (*Sallai, Periodica Polytechnica Electrical Engineering and Computer Science, 2024*), the principles of operation and performance of the Epic BenchTop and Epic Cardio label-free waveguide grating biosensors are descibed and their applications in various research areas are also discussed.

Fluidic flow plays important roles in colloid and interface sciences. Measuring adsorption, aggregation processes and living cell behavior under a fluidic environment with varied flow velocities in a parallel and high-throughput manner remains to be a challenging task. In our pubication (*Kovács et al. Journal of Colloid and Interface Science, 202*1), a method is introduced to monitor cell response to well-defined flow with varied velocities over an array of label-free resonant waveguide grating (RWG) based optical biosensors. The arrangement consists of a circular well with an array of biosensors at the bottom surface. By rotating the liquid over the biosensor array using a magnetic stirrer bar, flow velocities from zero to a predefined maximum can be easily established over different locations within the biosensor array as characterized in detail by numerical simulations. Cell adhesion and detachment measurements on an Arg-Gly-Asp (RGD) peptide functionalized surface were performed to demonstrate i) measurements at a wide range of simultaneous flow velocities over the same interface; ii) the possibility of parallel measurements at the same flow conditions in one run; and iii) the simple tuning of the employed range of flow velocities. Our setup made it possible to analyze the magnitude and rate of cell detachment at various flow velocities in parallel and determine the critical velocity and force where cells start to detach from the RGD motif displaying biomimetic surface.

Fluidic flow plays important roles in colloid and interface sciences. Measuring adsorption, aggregation processes and living cell behavior under a fluidic environment with varied flow velocities in a parallel and high-throughput manner remains to be a challenging task. We started to develop this method by miniaturizing the device which is able to measure two wells simultaneously.

Self-driven actions, such as motion, are fundamental characteristics of life. Today, intense research efforts are focusing on designing self-propelled motion in non-living systems to emulate organismic movement, utilizing entities like vesicles, solid particles, and droplets propelled at interfaces or within liquid phases. In this year, we employed surface sensitive surface-sensitive label-free optical waveguide sensors to in- depth investigate the kinetic changes in a self-assembled monolayer of the stearyl trimethyl ammonium chloride (STAC) surfactant on a substrate surface during the self-propelled motion of nitrobenzene droplets. The manusciprt in this topic is submitted to the Journal of Colloid and Interface Science journal.

Results using grating coupled interferometry (GCI):

Epigallocatechin-gallate (EGCG) is the main polyphenol ingredient of green tea. This compound is a strong antioxidant and oxidizes easily. Numerous studies demonstrated its beneficial effects on the human health, for example its anticancer and anti-inflammatory activity. In the body, EGCG is transported by serum albumin. EGCG easily oxidizes and the

interactions of the oxidized form presumably present significant differences. However, the presence of oxidized EGCG is usually neglected in the literature and its effects have not been investigated in detail. In our study, we applied the label-free grating coupled interferometry method that performs dual-channel measurements. The measured kinetic signal can be compensated with a signal of a reference channel at each measurement time. By testing both hydrophilic and hydrophobic platforms, we found that EGCG can bind to a wide range of surfaces. Exploiting the dual-channel referencing ability as well as the unique sensitivity and throughput of the employed label-free technique, the experiments revealed the specific interactions between bovine serum albumin (BSA) and EGCG/oxidized EGCG and determined the characteristic dissociation constant (Kd) of the binding equilibrium (*Peter et al. Analyst, 2020*).

In an other work, we measured the binding kinetics of Ni(II) ions to genetically modified flagellin layers and we demonstrate that: (1) Grating-Coupled Interferometry (GCI) is well suited to resolve the binding of ions, even at very low protein immobilization levels; (2) it supplies high quality kinetic data from which the number and strength of available binding sites can be determined, and (3) the rate constants of the binding events can also be obtained with high accuracy. Experiments were performed using a flagellin variant incorporating the C-terminal domain of the nickel-responsive transcription factor NikR. GCI results were compared to affinity data from titration calorimetry. We found that besides the low-affinity binding sites characterized by a micromolar dissociation constant (Kd), tetrameric FliC-NikRC molecules possess high-affinity binding sites with Kd values in the nanomolar range. GCI enabled us to obtain real-time kinetic data for the specific binding of an analyte with molar mass as low as 59 Da, even at signals lower than 1 pg/mm^2 (*Jankovics et al. Scientific Reports, 2020*).

The label-free interaction analysis of macromolecules and small molecules has increasing importance nowadays, both in diagnostics and therapeutics. In the blood vascular system, human serum albumin (HSA) is a vital globular transport protein with potential multiple ligand binding sites. Characterizing the binding affinity of compounds to HSA is essential in pharmaceutics and in developing new compounds for clinical application. Aryltetralin lignans from the roots of *Anthriscus sylvestris* are potential antitumor therapeutic candidates, but their molecular scale interactions with specific biomolecules are unrevealed. Here, we applied the GCI biosensing method with a polycarboxylate-based hydrogel layer with immobilized HSA on top of it. With this engineered model surface, we could determine the binding parameters of two novel aryltetralin lignans, deoxypodophyllotoxin (DPT), and angeloyl podophyllotoxin (APT) to HSA. Exploiting the multi-channel referencing ability, the unique surface sensitivity, and the throughput of GCI, we first revealed the specific biomolecular interactions. Traditional label-free kinetic measurements were also compared with a novel, fast way of measuring affinity kinetics using less sample material (repeated analyte pulses of increasing duration (RAPID)). Experiments with well-characterized molecular interactions (furosemide to carbonic-anhydrase (CAII) and warfarin, norfloxacin to HSA) were performed to prove the reliability of the RAPID method. In all investigated cases, the RAPID and traditional measurement gave similar affinity values (Figure 3). These results could be also adapted to other biomolecules and applications where sample consumption and the rapidity of the measurements are critical (*Péter et al. International Journal of Biological Macromolecules, 2023*).

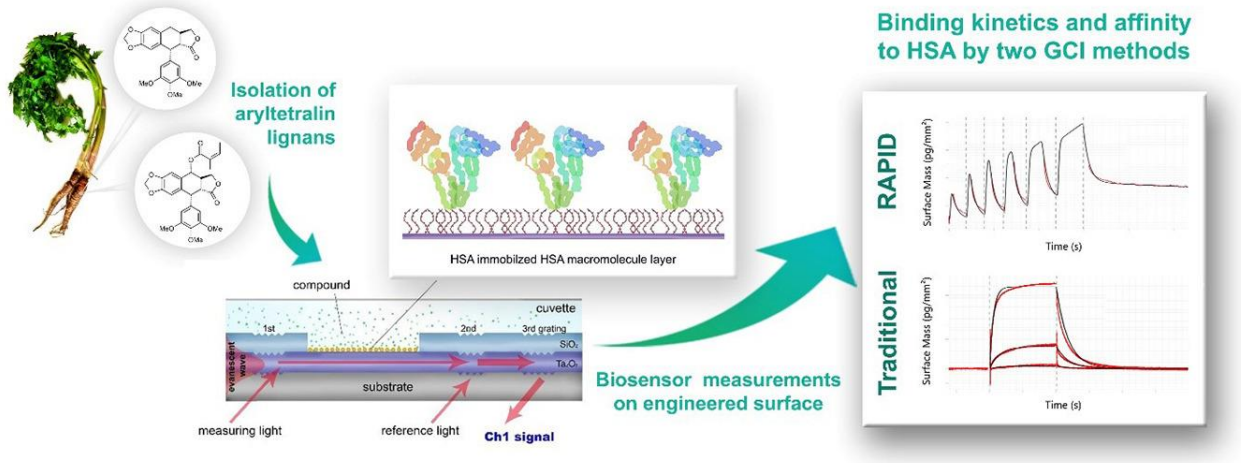

Figure 3. *Schematic illustration of the applied methodology.*

We started to test antimicrobial peptides by the GCI method as well.

Since February 2024, we have also started to perform experiments with a new technique, mology. Mology is the newest biosensor technique in our laboratory. It is worth mentioning as a next-generation label-free optical biosensor that may open up new perspectives for kinetic measurements. In mology, detection is based on probing the mology by laser light coupled into a mono-mode waveguide film that constitutes the sensor chip. The mology is defined as a synthetic hology, which is a precise nanopatterned assembly of receptor molecules. The main advantage of this method is that it can discriminate specific binding from non-specific ones, which otherwise always degrades biosensing signals. We plan to exploit this advantage in basic cell research and measure receptor-ligand functions and cell adhesion.

WP2–Nano- and microstructured functional coatings for controlling cellular behavior

The main objective was to fabricate functional surfaces with well-controlled chemical, mechanical, and topological properties. The creation and in-depth characterization of functional nanofilms of polymers and matrix proteins. The above functional objects will be deposited in a highly controlled and reproducible manner. The FluidFM BOT technology will be exploited to pattern large areas with multiple functionalities. The mechanical properties will be characterized by AFM and FluidFM.

Micropatterning of living single cells and cell clusters over millimeter–centimeter scale areas is of high demand in the development of cell-based biosensors. Micropatterning methodologies require both a suitable biomimetic support and a printing technology. In our study, we presented the micropatterning of living mammalian cells on carboxymethyl dextran (CMD) hydrogel

layers using the FluidFM BOT technology (Figure 4). In contrast to the ultrathin (few nanometers thick in the dry state) CMD films generally used in label-free biosensor applications, we developed CMD layers with thicknesses of several tens of nanometers in order to provide support for the controlled adhesion of living cells (*Saftics et al. Langmuir, 2019*).

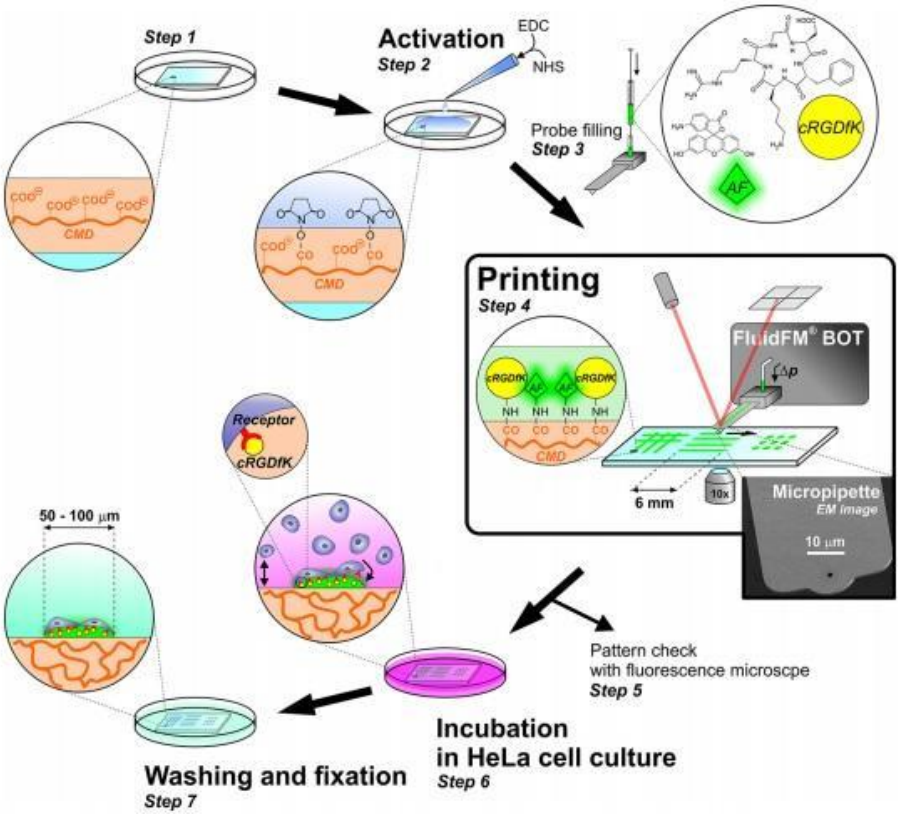

Figure 4. *Method used for cell adhesion experiments on the cRGDfK-printed CMD surface, where microprinting was performed with the FluidFM technique.*

In 2020, we published our book chapter with the title of 'Dextran-based hydrogel layers for biosensors' in Nanobiomaterial Engineering (*Saftics et al. 2020*). In this case study, we focused on the analysis of carboxymethyl dextran (CMD) layers developed for waveguide-based label-free optical biosensor applications. Here, the examination methodologies both under dry and aqueous conditions as well as testing of antifouling abilities were also presented (Figure 5).

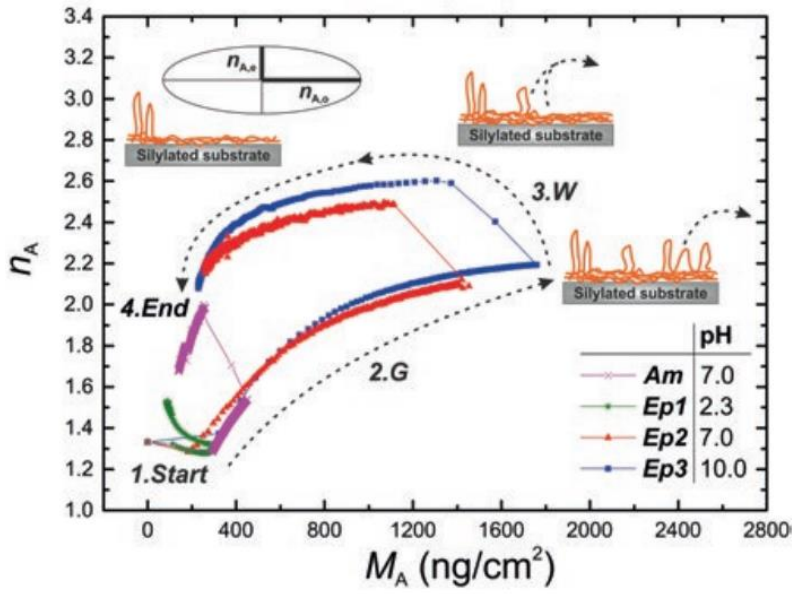

Figure 5. *Refractive index of CMD-ut layer as a function of deposited surface mass density. The curves represent different experimental conditions revealing the dependence of CMD layer structure during its formation on the applied silane precoating and pH of the grafting solution. The time-related direction of the measurements is indicated by the dashed arrows (G indicates the grafting, W the washing section of experiments). The inset table represents the different experimental conditions (Am, aminosilylated; Ep, epoxysilylated surfaces). The schemes above the curves illustrate the alteration of CMD layer nanostructure at the different experimental phases*

Then, we turned our attention to bacterial cell adhesion as well. Bacteria repellent surfaces and antibody-based coatings for bacterial assays have shown a growing demand in the field of biosensors, and have crucial importance in the design of biomedical devices, and mammalian-bacteria co-cultures. We described an OWLS-based method supporting the development of bacteria-repellent surfaces and characterized the layer structures and affinities of different antibody-based coatings for bacterial assays (Figure 6). The best performance in the biosensor measurements was achieved by employing a polyclonal antibody in combination with protein A-based immobilization and PAcrAM-P blocking of nonspecific binding. Using this setting, a surface sensitivity of 70 cells/mm^2 was demonstrated (*Farkas et al. Biosensors, 2022*).

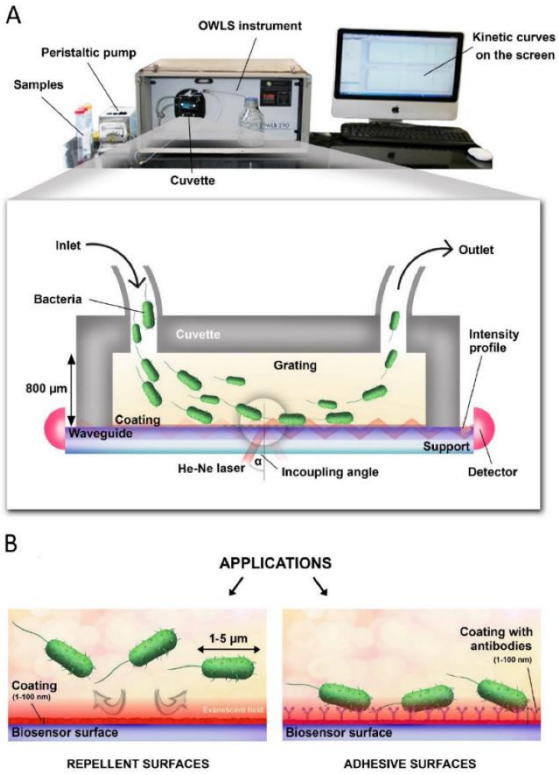

Figure 6. *(A) Cross-sectional view of the OWLS cuvette and the basics of optical detection. Laser light is coupled into an optical waveguide layer by a surface grating where it propagates by total internal reflection to a photodetector placed at the end of the waveguide. Adsorbing bacteria shift the resonant angle (α). (B) OWLS is an ideal tool for testing and developing both bacteria repellent and bacteria adhesive surfaces.*

Handling of picoliter-to-nanoliter-scale volumes and objects has increasing importance in life sciences. This is the volume scale of cell extractions and individual living cells. in our work, we introduced a method of generating a picoliter-scale device by direct writing of picowell arrays on a ZEONOR™ copolymer surface with high-energy medium-mass ion microbeam. Arrays of various microstructures were written in the sample using a microbeam of 10.5 MeV N4+ ions at various implanted ion fluences. The best array was obtained by implantation of annuli of 10 and 11 µm of inner and outer diameters with a fluence of 7.8×1012 ions/cm^2 (*Bányász et al. Applied Physics Letters, 2023*).

In another work, the adhesion of preosteoblastic cell line MC3T3-E1 on polysaccharide-based multilayers was evaluated using a resonant waveguide grating (RWG) based optical biosensor and digital holographic microscopy. The latter method was engaged to investigate long-term cellular behavior on the fabricated multilayers. The (PDADMAC/heparin) films were proved to be the most effective in inducing cellular adhesion. The cell attachment to chitosan/heparin–based multilayers was negligible. It was found that efficient adhesion of the cells occurs onto homogeneous and rigid multilayers (PDADMAC/heparin), whereas the macroion films forming “sponge-like” structures (chitosan/heparin) are less effective, and could be employed when reduced adhesion is needed. Polysaccharide-based multilayers can be considered versatile systems for medical applications. This project was published in the prestigeous International Journal of Biological Macromolecules (*Wasilewska et al. International Journal of Biological Macromolecules, 2023*).

WP3- Adhesion studies on homogeneous and patterned functional supports

The developed sensor units will be used for characterizing the kinetics of extracellular vesicle and cellular adhesion on the WP2 developed model surfaces and to detect cell refractive index variations at the nanometer lengthscale in single cells. The results will be compared with complementary techniques based on mechanical (FluidFM BOT) and fluorescence signal generation in order to connect the measured signals with biologically relevant properties to significantly extend the capabilities of label-free optical sensors.

The field of extracellular vesicles (EVs) is an exponentially growing segment of biomedical sciences. With scientists from Semmelweis University and ELTE, we reported a quick, reliable, and sensitive test that may fill an existing gap in EV standardization. When using the optimized lipid assay reported in our study, EV lipid measurements can be more reliable than protein-based measurements (Figure 7). Furthermore, this novel assay is almost as sensitive and as easy as measuring proteins with a simple BCA test (*Visnovitz et al., Journal of Extracellular Vesicles, 2019*).

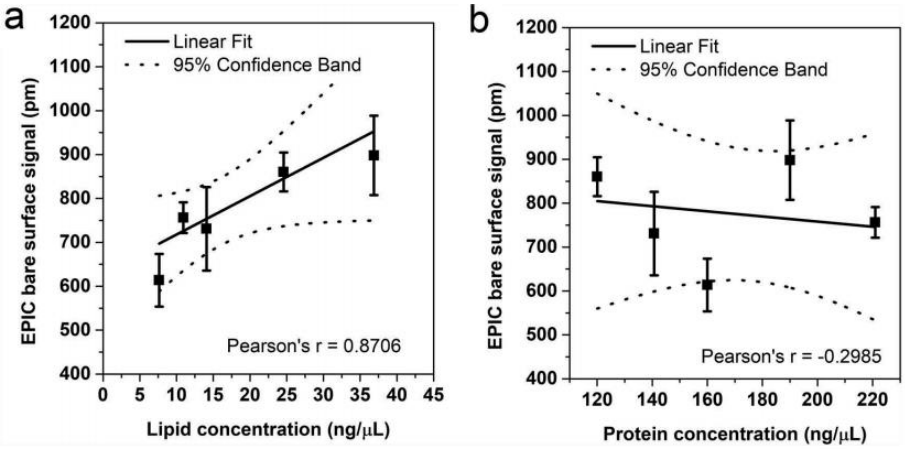

***Figure 7**. Correlation between the lipid and protein concentrations of EVs with the "bare surface signal" of an EPIC optical biosensor. Correlation between the EPIC "bare surface signal" and either the lipid concentration (a) or the protein concentration (b). Small EVs were isolated from serum-free conditioned medium of THP-1 cells. Data points are the average of three replicates; error bars represent SD.*

In another topic, phase holographic imaging was used to monitor the time-dependent behavior of cancerous HeLa cells. After monitoring the assembly of epithelial Vero monolayer on a gelatin-coated surface, HeLa cells were seeded on top of the monolayer, and their transmigration was observed (Figure 8). This method is label-free and non-toxic to cells and enables the visualization of living cells in real-time, and analyzes their parameters such as motility and morphology. HeLa cells seeded on the tight Vero monolayer (100 % confluency) were observed for 24 hours, and a 60-minute time period was selected for further analysis that showed the difference in cellular parameters between non-invasive and invasive HeLa cells in

the observed time frame. Our investigations revealed that invasive HeLa cells have reduced area and optical volume compared to noninvasive HeLa cells, corresponding to the phase shift detected in transmigration events (*Nagy et al. IEEE Xplore, 2020*).

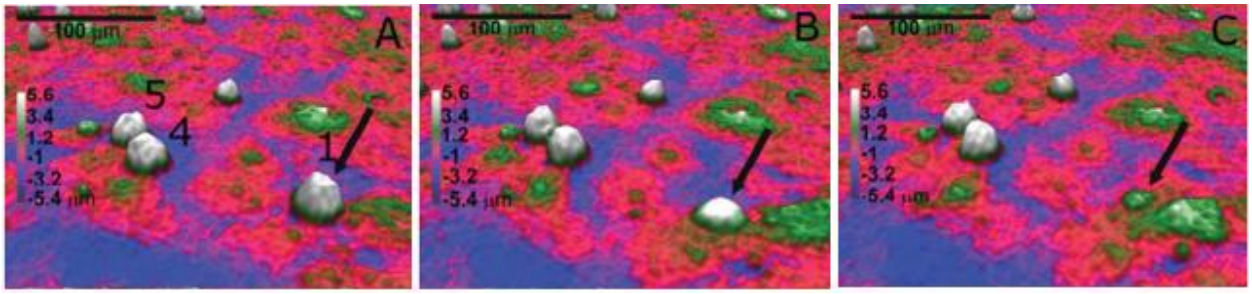

***Figure 8**. Intravasation of a HeLa cell seeded on top of the Vero monolayer. (A, B, C) The invasive Hela cell (1) marked with black arrow embadded itself into the underlying Vero monolayer. The 4 and 5 are non-invasive HeLa cells.*

Optimizing nanostructures in aqueous media is challenging. The purpose of our investigation is to accurately measuring cell adhesion, surface nanostructures can be measured, characterized, and precisely tuned. This technique can provide a link between chemical structure and biological activity, as opposed to conventional analytical methods. In our work, we optimized cell-adhesive behavior of poly(L-lysine)-graft-poly(ethylene glycol)-azide (PPN_3) + dibenzocyclooctyne -Arg-Gly-Asp (DBCO-RGD) polymer layers with capturing click chemistry by cell adhesion measuring. We demonstrated a stabile layer up to one month can be created, which can be a good alternative to PP-RGD polymer at 10 µM concentration or above, that shows a corresponding cell adhesion signal on RWG biosensor. It is a fast reaction, capable of forming a surface coating with sufficient cell adhesion on PPN_3 during 5 minutes. We intend to publish these results until the end of 2024.

WP4- Cells under external stimuli (chemical, biological and mechanical)

Various compounds (such as anticancer agents, exosomes, other cells, etc.) were added to the cells and tissue models and their responses will be monitored. Immune (B), endothelial, and cancer cells were the main model systems.

Our aim was to investigate the effects of antioxidant superoxide dismutase (SOD)-like enzyme substituting metal complex systems on cell processes by applying novel, label-free biosensor method. SALEN- and SALAN-based complexes with catalytically active metal centers are very promising small molecules to be utilized as part of antioxidant therapies. In our novel study, we discussed a modified SALAN-type molecule armed with two phosphonate groups that significantly increase its water solubility and aid to furnish mono- or dinuclear complexes with Cu2+ ions. We reported that phosphonate groups affect coordination of Cu(II) to a SALAN ligand and increase water solubility. Furthermore, SOD-like activity and overall cell toxicity are both affected by Cu/ligand ratio. The SOD-mimc 1Cu/ligand complex is well tolerated by living cells (*Székács et al. Chemico-Biological Interactions, 2019*).

Constituting the inner lining of vessel walls, endothelial cells are the key players in various physiological processes, moreover, they are the first to be exposed to most drugs currently used. However, to date, there is still no appropriate technology for the label-free, real-time and high-throughput monitoring of endothelial function. To this end, we developed an optical biosensor-based endothelial label-free biochip (EnLaB) assay that meets all the above requirements. Using our EnLaB platform, we screened a set of plasma serine proteases as possible endothelial cell activators, and first identified the endothelial cell activating function of three important serine proteases – namely kallikrein, C1r and mannan-binding lectin-associated serine-protease 2 (MASP-2) – and verified these results in well-established functional assays (Figure 10). EnLaB proved to be an effective tool for revealing novel cellular mechanisms as well as for the high-throughput screening of various compounds on endothelial cells (*Debreczeni et al. Scientific Reports, 2020*).

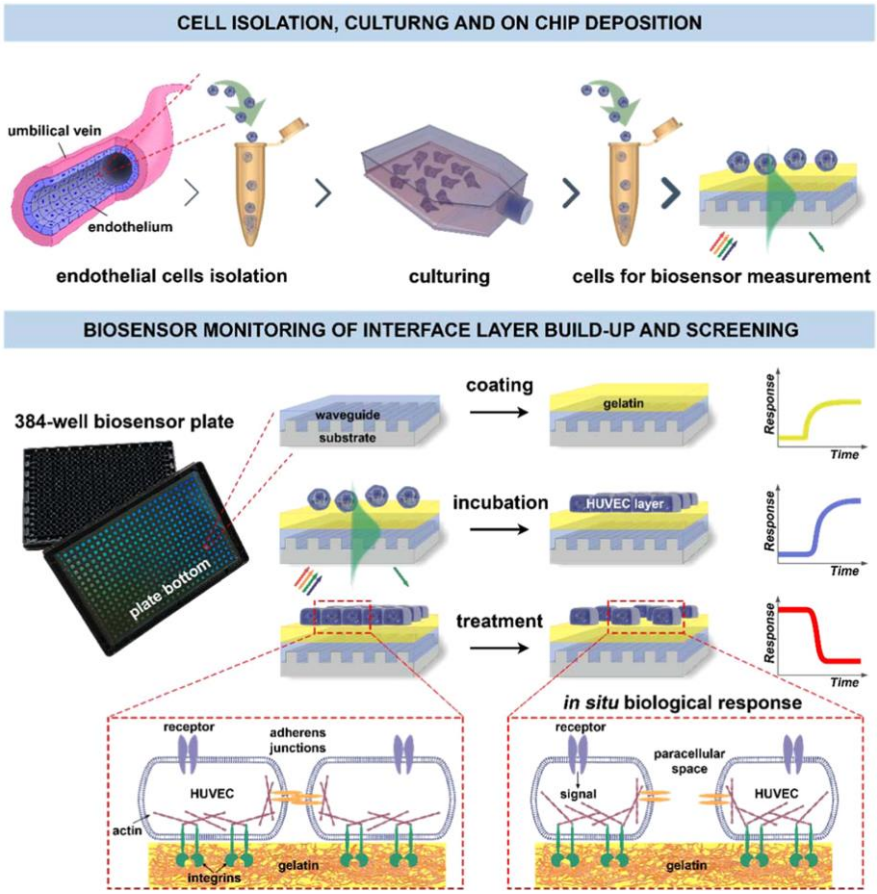

Figure 10*. Schematic overview of the proposed EnLaB measurement setup. Upper part: cell preparation steps (primary cell isolation, culturing, transferring onto the sensor chip surface). The lower part illustrates the steps of the biosensor measurements and typically obtained biosensor responses (the detected shifts in the resonant wavelength): coating of the chip with gelatin, following with cell attachment to the gelatin surface, and subsequent cell treatment by the studied molecular compounds (screening). Illustration of the biological effect of the treatment is highlighted in dashed boxes.*

The glycocalyx is a cell surface sugar layer of most cell types that greatly influences the interaction of cells with their environment. Interestingly, cancer cells have a thicker glycocalyx layer compared to healthy cells, but to date, there has been no consensus in the literature on the exact role of cell surface polysaccharides and their derivatives in cellular adhesion and

signaling. We investigated the effect of enzymatic digestion of specific glycocalyx components on cancer cell adhesion to RGD (arginine–glycine–aspartic acid) peptide motif displaying surfaces. High resolution kinetic data of cell adhesion was recorded by the surface sensitive label-free resonant waveguide grating (RWG) biosensor, supported by fluorescent staining of the cells and cell surface charge measurements (Figure 11). We found that intense removal of chondroitin sulfate (CS) and dermatan sulfate chains by chondroitinase ABC reduced the speed and decreased the strength of adhesion of HeLa cells. In contrast, mild digestion of glycocalyx resulted in faster and stronger adhesion. Control experiments on a healthy and another cancer cell line were also conducted, and the discrepancies were analysed. We developed a biophysical model which was fitted to the kinetic data of HeLa cells. Our experimental results and modelling demonstrated that the chondroitin sulfate and dermatan sulfate chains of glycocalyx have an important regulatory function during the cellular adhesion process, mainly controlling the kinetics of integrin transport and integrin assembly into mature adhesion sites. Our results potentially open the way for novel type of cancer treatments affecting these regulatory mechanisms of cellular glycocalyx (*Kanyo et al. Scientific Reports, 2020*).

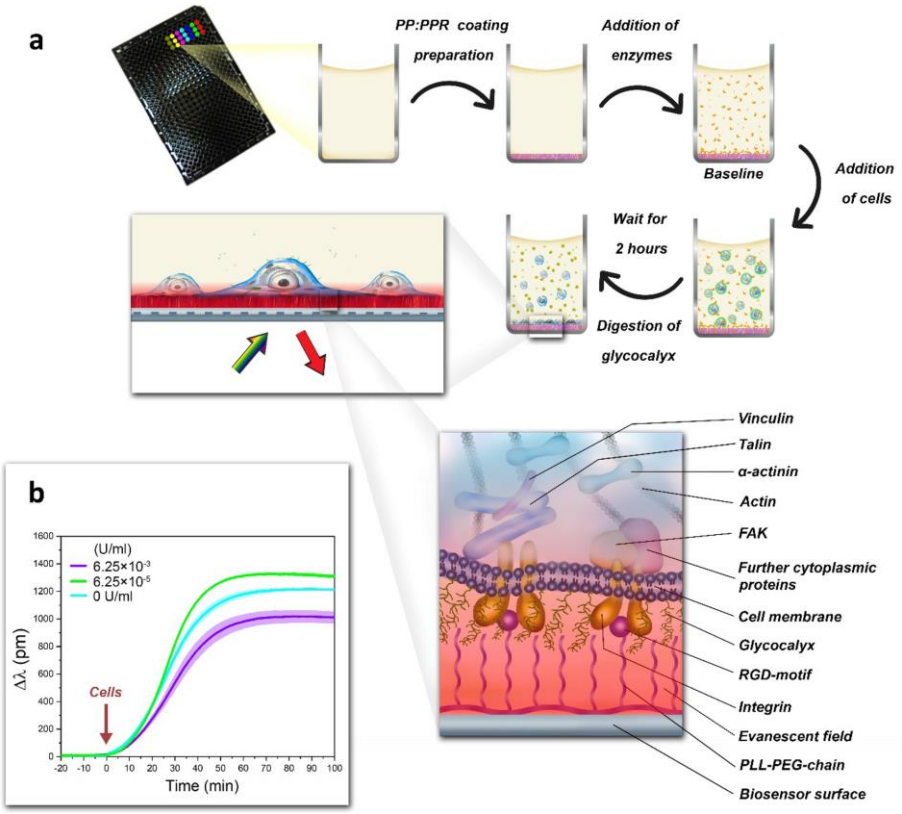

Figure 11. *Schematics of RWG measurements of cell adhesion kinetics on the polymer coated biosensor surfaces. (a) The adhesion kinetics of cells were real-time monitored using the label-free optical biosensor. First, the PP: PPR copolymer coating was prepared on the sensor surfaces, and the ChrABC enzyme at different concentrations was added to the wells. After recording a baseline HeLa cells were pipetted into the biosensor wells (0 min). The cell adhesion was monitored for 100 min. The schematic illustration of the adhered cells in the biosensor wells and the cellular components are also shown in the magnified parts. The surface localized evanescent optical field is illustrated as red shadow. (b) Representative cell adhesion kinetic curves on 50% PP: PPR copolymer surface.*

In the following study, a high spatial resolution label-free optical biosensor was employed to monitor the adhesivity of cancer cells both at the single-cell and population level. Population-level distributions of single-cell adhesivity were first recorded and analyzed when ChrABC enzyme was added to the adhering cells. The presented results open up new directions in glycocalyx-related cell adhesion research and in the development of more meaningful targeted cancer treatments affecting adhesion (*Kanyo et al. Matrix Biology Plus, 2022*). After that, we *in situ* monitored the cellular uptake of gold NPs─functionalized with positively charged alkaline thiol (TMA)─into adhered cancer cells with or without preliminary glycocalyx digestion as well. Our results raise the hypothesis that cellular uptake of 2–4 nm positively charged NPs is facilitated by glycoprotein and glycolipid components of the glycocalyx but inhibited by proteoglycans (*Peter et al. ACS Applied Bio Materials, 2022*). In our experiments we injected gold nanoparticles into HeLa cells as well. Furthermore, we continued the in situ monitoring of the cellular uptake of gold NPs─functionalized with positively charged alkaline thiol (TMA)─into adhered cancer cells with or without preliminary glycocalyx digestion. This time, we observed this phenomenon on a single cell level (not on a cell population level). We plan to publish our results next year.

Fibronectin is an extracellular matrix component that plays a significant role in many physiological processes, such as cell adhesion, growth, differentiation, and migration. In our study, we revealed the interaction between this important protein and the widely studied natural active substance green tea polyphenol epigallocatechin-gallate (EGCG) and its oxidized form. Furthermore, we investigated the kinetics of cancer cell adhesion on the polyphenol-treated fibronectin coatings. We applied a high-throughput, label-free optical biosensor capable of monitoring the above processes in real time with an excellent signal-to-noise ratio (*Peter et al. Materials Advances, 2022*).

In another work, the cytotoxic effects of Roundup Classic herbicide and its components (for example glyphosate) were also studied on different cell lines by biochemical and flow cytometric assays (Oláh et al.. Toxicology Reports, 2022). Furthermore, we investigated how extended the inhibitory effect observed for herbicide glyphosate on the integrin αvβ3 is in terms of other RGD integrins and other structurally or metabolically related derivatives of glyphosate (*Gémes et al. International Journal of Molecular Sciences, 2022*).

In our work (Kliment et al. Biosensors and Bioelectronics, 2021) a resonant waveguide grating (RWG)-based label-free optical biosensor technique has been applied for real-time monitoring of the integrated responses of primary human tonsillar B cells initiated by B cell receptor (BCR) and modified by FcγRIIb and CR1 engagement. The BCR-triggered biosensor responses of resting and activated B cells were revealed to be specific and dose-dependent, in some cases with strong donor dependency. Targeted inhibition of Syk attenuated the label-free biosensor response upon BCR stimulation. Indifferent protein human serum albumin (HSA) did not interfere with the recorded signal to BCR stimulation.

In the past decade, extracellular vesicles (EVs) have attracted substantial interest in biomedicine. With progress in the field, we have an increasing understanding of cellular responses to EVs. In our work, we described the direct nanoinjection of EVs into the cytoplasm of single cells of different cell lines. By using robotic fluidic force microscopy (robotic

FluidFM), nanoinjection of GFP positive EVs and EV-like particles into single live HeLa, H9c2, MDA-MB-231 and LCLC-103H cells proved to be feasible (Figure 12). This injection platform offered the advantage of high cell selectivity and efficiency. The nanoinjected EVs were initially localized in concentrated spot-like regions within the cytoplasm. Later, they were transported towards the periphery of the cells. Based on our proof-of-principle data, robotic FluidFM is suitable for targeting single living cells by EVs and may lead to information about intracellular EV cargo delivery at a single-cell level (*Kovács et al. Journal of Extracellular Vesicles, 2023*).

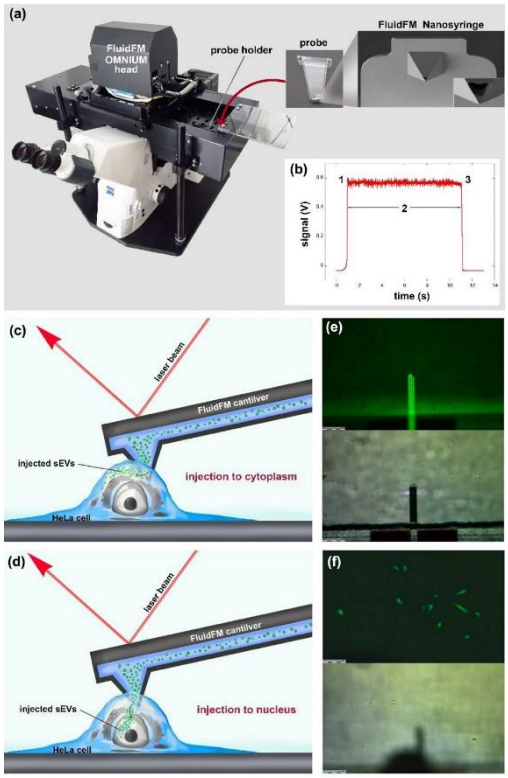

Figure 12. *Summary of the FluidFM measurements. (a) Photo of the FluidFM appliance, probe and the FluidFM Nanosyringe. (b) Change of the voltage signal during injection of a live cell. (c) Schematic illustration of the injection of sEVs to the cytoplasm by FluidFM. (d) Schematic illustration of the injection of sEVs to the nucleus by FluidFM. (e) Microscopic image of the FluidFM cantilever in fluorescent mode (upper part) and bright field mode (bottom part). (f) Microscopic image of the cells in fluorescent mode (upper part) and bright field mode (bottom part).*

WP5- Mathematical modeling and numerical simulations

Optical and biophysical theoretical models for interpreting the measured signals and the kinetics of changes will be developed. This way we will deeper understand the biophysical and biological background of cellular adhesion and cell refractive index variations. Numerical simulations will be also used to model the electromagnetic wave propagations in the waveguides, the effect of fluid flow on the cells, and the pulling forces over the individual cells and compact cell monolayers.

Optical Waveguide Lightmode Spectroscopy (OWLS) is widely applied to monitor protein adsorption, polymer self-assembly, and living cells on the surface of the sensor in a label-free manner. Typically, to determine the optogeometrical parameters of the analyte layer (adlayer), the homogeneous and isotropic thin adlayer model is used to analyze the recorded OWLS data. However, in most practical situations, the analyte layer is neither homogeneous nor isotropic. Therefore, the measurement with two waveguide modes and the applied model cannot supply enough information about the parameters of the possible adlayer inhomogeneity and anisotropy. Only the so-called quasihomogeneous adlayer refractive index, layer thickness, and surface mass can be determined. In the present work, we construct an inhomogeneous adlayer model. In our model, the adlayer covers the waveguide surface only partially and it has a given refractive index profile perpendicular to the surface of the sensor (Figure 13). Using analytical and numerical model calculations, the step-index and exponential refractive index profiles are investigated with varying surface coverages from 0 to 100%. The relevant equations are summarized and three typically employed waveguide sensor structures are studied in detail (*Kovács and Horvath. Journal of Sensors, 2019*).

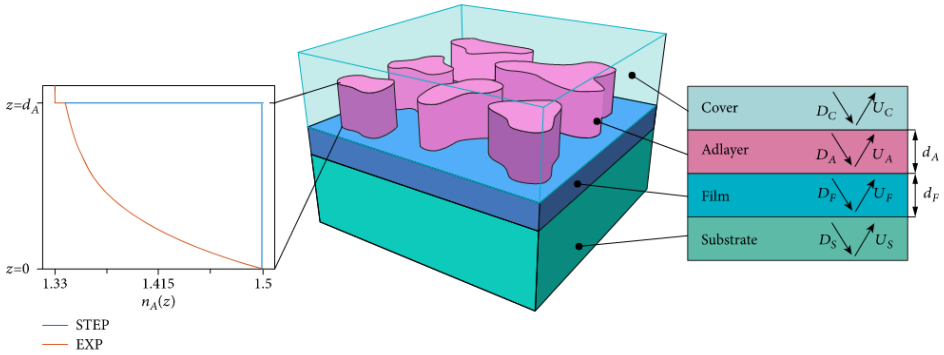

***Figure 13**. The structure of the modeled OWLS waveguide chips with inhomogeneous adlayer. The modeled multilayered assembly consists of 4 layers: substrate, waveguide film, adlayer, and cover.*

Biophysical model development for refractive index variations caused by living cells, the detailed study of the kinetics of changes using a program based on an integrator routine. In this period, numerical simulations and analytical calculations were performed to support the design of grating-coupled planar optical waveguides for biological sensing. In our article (*Kovács et al. Scientific Reports, 2021*), where near cut-off and far from cut-off modes were investigated (Figure 14), and their characteristics and suitability for sensing are compared. The numerical simulations revealed the high sensitivity of the guided mode intensity near the cut-off wavelength for any refractive index change along the waveguide. Consequently, it is sufficient to monitor the intensity change of the near cut-off sensing mode, which leads to a simpler sensor design compared to those setups where the resonant wavelength shift of the guided mode is monitored with high precision.

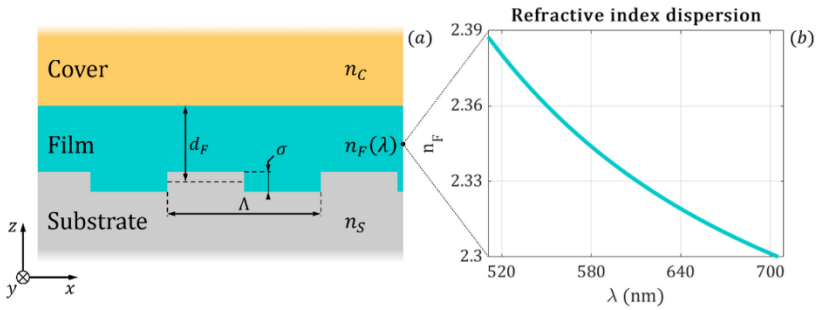

Figure 14. *(a) The cross-section of the modeled corrugated waveguide. The electromagnetic simulations apply Floquet boundary conditions along the x- and y-axis and open boundaries with Floquet ports in the vertical z direction. (b) The frequency dependence of the Nb2O5 film refractive index*.

We showed the existence of chemical resonance, chemical beats, and frequency locking phenomena in periodically forced pH oscillatory systems (sulfite–hydrogen peroxide and sulfite–formaldehyde–gluconolactone pH oscillatory systems, Figure 15). Periodic forcing was realized by a superimposed sinusoidal modulation on the inflow rates of the reagents in the continuous-flow stirred tank reactor. The dependence of the time period of beats follows the relation known from classical physics for forced physical oscillators. Our developed numerical model describes qualitatively the resonance and beat phenomena experimentally revealed. Application of periodic forcing in autonomously oscillating systems can provide new types of oscillators with a controllable frequency and new insight into controlling irregular chemical oscillation regimes.
Moreover, numerical simulations were performed on resonant waveguide gratings and the intensity and resonance peak position interrogation were compared in detail (*Lawson et al. The Journal of Physical Chemistry Letters, 2020*).

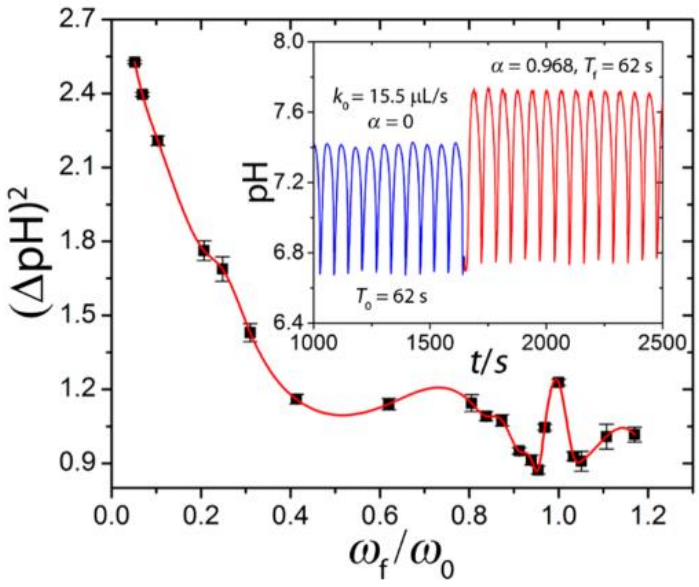

Figure 15. *Chemical resonance curve in the sulfite−hydrogen peroxide pH oscillatory system using a sinusoidal periodic forcing of the inflowrate of the reagents (k0 = 15.5 µL/s, and α = 0.968). ΔpH is the peak-to-peak amplitude of the oscillation. The inset shows the oscillations in the unforced case (blue line) with a time period of 62 s and the oscillations when a sinusoidal periodic forcing is applied (red line) with the natural frequency of the oscillatory system. Close to the natural frequency, where beat phenomena appeared, the amplitude of the oscillations was calculated as an average amplitude corresponding to the one period of beat.*

We studied the cellular adhesion maturation of epithelial Vero monolayers by measuring single-cell force-spectra with FluidFM. We found that HeLa cells adhere significantly stronger to the tight Vero monolayer than cells of the same origin. Moreover, the mechanical characteristics of Vero monolayers upon cancerous HeLa cell influence were recorded and analyzed (*Nagy et al. European Journal of Cell Biology, 2022*). We have also experimentally introduced a novel parameter, the spring coefficient of a whole cell.

Fluidic force microscopy (FluidFM) fuses the force sensitivity of atomic force microscopy with the manipulation capabilities of microfluidics by using microfabricated cantilevers with embedded fluidic channels. This innovation initiated new research and development directions in biology, biophysics, and material science. To acquire reliable and reproducible data, the calibration of the force sensor is crucial. The hollow FluidFM cantilevers contain a row of parallel pillars inside a rectangular beam. The precise spring constant calibration of the internally structured cantilever is far from trivial, and existing methods generally assume simplifications that are not applicable to these special types of cantilevers. In addition, the Sader method, which is currently implemented by the FluidFM community, relies on the precise measurement of the quality factor, which renders the calibration of the spring constant sensitive to noise. In our publised study (*Bonyár et al. Microsystems and Microengineering, 2024*), the hydrodynamic function of these special types of hollow cantilevers was experimentally determined with different instruments. Based on the hydrodynamic function, a novel spring constant calibration method was adapted, which relied only on the two resonance frequencies of the cantilever, measured in air and in a liquid. Based on these results, our proposed method can be successfully used for the reliable, noise-free calibration of hollow FluidFM cantilevers.

FluidFM BOT, which can perform up to 30-40 Single-Cell Force-Spectroscopy (SCFS) measurements per day exceeding previous SCFS acquisition methods. The user software of the FluidFM BOT allows the user to measure the inverse optical lever sensitivity (InvOLS) and spring constant (k) of the applied FluidFM micropipette cantilevers used for SCFS recording, which parameters directly scale the obtained voltage values from the twosegmented photodetector. However, the user software miscalculates the InvOLS values and does not allow exporting measurement parameters Fmax, Emax, and Dmax, which are characteristic of individual SCFS Force-Distance (FD) curves. Also, exporting individual FD-curve plots is not possible. In our symposium article (*Nagy et al. IEEE SITME, 2021*) we show a software specially developed in Matlab for the correct acquisition of InvOLS values, plotting and exporting of FD-curves and their parameters. The software allows users to interfere, but it is partially automated to enable smooth data evaluation procedures. The software is available free of charge at the nanobiosensorics.com website.

Selecting and isolating various cell types is critical in many applications, including immune therapy, regenerative medicine, and cancer research. Usually, these selection processes involve some labeling or another invasive step potentially affecting cellular functionality or damaging the cell. In our recent article (*Kovács et al. Scientific Reports, 2024*), we first introduced an optical biosensor-based method capable of classification between healthy and numerous cancerous cell types in a label-free setup. In this work, we presented high classification accuracy based on the monitored single-cell adhesion kinetic signals. We developed a high-throughput data processing pipeline to build a benchmark database of ~ 4500 single-cell adhesion measurements of a normal preosteoblast (MC3T3-E1) and various cancer (HeLa, LCLC-103H, MDA-MB-231, MCF-7) cell types. Several datasets were used with different cell-type

selections to test the performance of deep learning-based classification models, reaching above 70–80% depending on the classification task. Beyond testing these models, we aimed to draw interpretable biological insights from their results; thus, we applied a deep neural network visualization method (grad-CAM) to reveal the basis on which these complex models made their decisions. Our proof-of-concept work demonstrated the success of a deep neural network using merely label-free adhesion kinetic data to classify single mammalian cells into different cell types. We propose our method for label-free single-cell profiling and *in vitro* cancer research involving adhesion. The employed label-free measurement is noninvasive and does not affect cellular functionality. Therefore, it could also be adapted for applications where the selected cells need further processing, such as immune therapy and regenerative medicine. Of note, an additional manuscript was just accepeted for publication in *ACS Sensors*, where we extended the investigation for more cell types and reached better performance by employing the spatial distribution of adhesion contact kinetics.

Review type works (relevant for all WPs)

All together we published **6 review papers** in respected journals about our research topics during the project period.

We publised a review article, where we summarized the most important theoretical considerations for obtaining reliable information from measurements taking place in liquid environments and, hence, with layers in a hydrated state. A thorough treamtment of the various kinetic and structural quantities obtained from evaluation of the raw label-free data are provided. Such quantities include layer thickness, refractive index, optical anisotropy (and molecular orientation derived therefrom), degree of hydration, viscoelasticity, as well as association and dissociation rate constants and occupied area of subsequently adsorbed species. To demonstrate the effect of variations in model conditions on the observed data, simulations of kinetic curves at various model settings are also included (Figure 16). Based on our own extensive experience with optical waveguide lightmode spectroscopy (OWLS) and the quartz crystal microbalance (QCM), we have developed dedicated software packages for data analysis, which are made available to the scientific community alongside this paper (*Saftics et al. Advances in Colloid and Interface Science, 2021*).

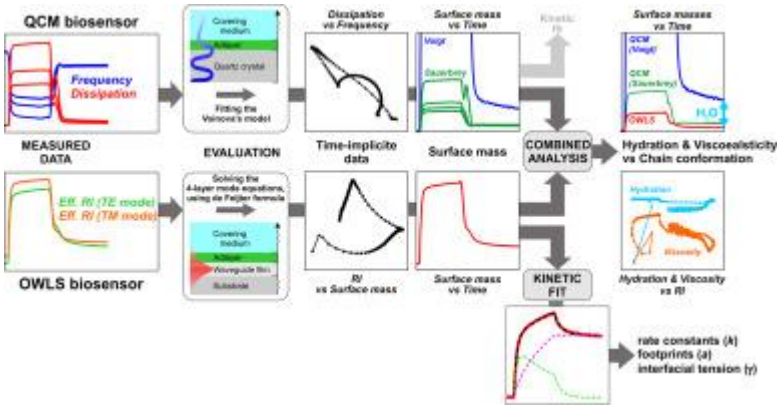

***Figure 16**. Based on our own extensive experience with optical waveguide lightmode spectroscopy (OWLS) and the quartz crystal microbalance (QCM), we have developed dedicated software packages for data analysis*

The quantitative and precise measurement of the adhesion strength of living cells is critical, not only in basic research but in modern technologies, too. Several techniques have been developed or are under development to quantify cell adhesion. All of them have their pros and cons, which has to be carefully considered before the experiments and interpretation of the recorded data. Our review provides a guide to choose the appropriate technique to answer a specific biological question or to complete a biomedical test by measuring cell adhesion (*Ungai-Salánki et al. Advances in Colloid and Interface Science, 2019*).

During this period, our aim was to summarize the current state of methods for label-free identification and functional characterization of leukocytes with biosensors and novel single-cell techniques in a review article. The emphasis is on techniques on the characterizations of single cells with special attention to surface-sensitive technologies. Recent developments highlighted the importance of small cell populations and individual cells both in health and disease. Nonetheless, techniques capable of analyzing single cells offer a promising tool for therapeutic approaches where the characterization of individual cells is necessary to estimate their clinical therapeutic potential (Szittner et al. Advances in Colloid and Interface Science, 2022). A Nature spotlight article was also published on the single-cell transcriptomic profiling method using picoliter scale single-cell cytoplasmic biopsies instead of complete cell lysis (*Horvath. Cell Reports Methods, 2022*).

In our review article, our aims were to to give an overview of the present status of label-free biosensors in bacteria monitoring (Figure 17), and to summarize potential novel directions with biological relevance to initiate future development in the field. Optical, mechanical, and electrical sensing technologies are all discussed with their detailed capabilities in bacteria monitoring (*Péter et al. Biosensors, 2022*).

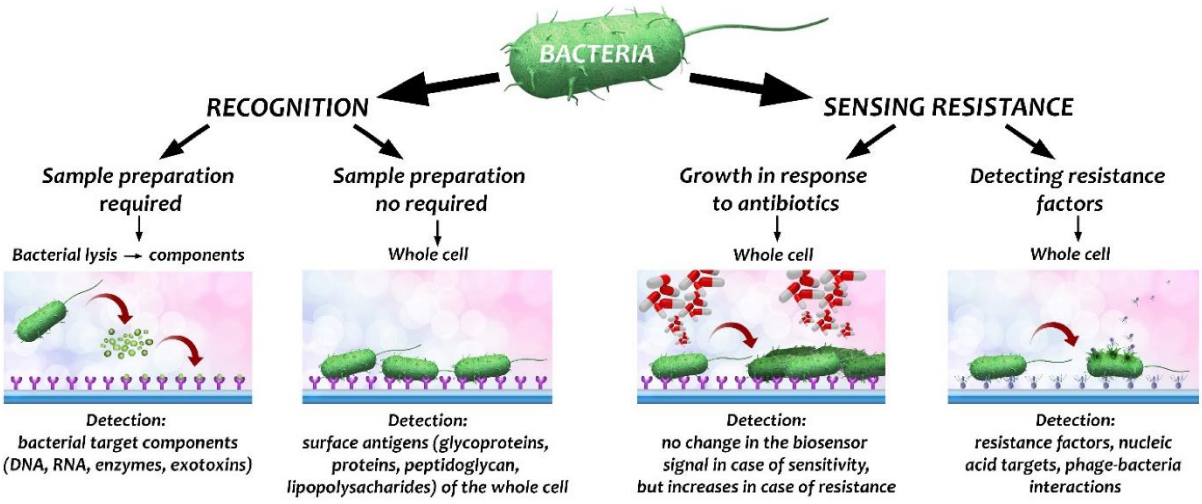

***Figure 17.** Summary of the strategies of recognition of bacteria and ways of resistance sensing using biosensors. Sample preparation may be needed to lyse the bacteria (or otherwise disrupt them) to liberate the target bacterial components (first column); and preparation-free whole cell-based assays are in the second column. Few biosensors can sense antibiotic resistance as well. There are two possibilities: measuring and monitoring the growth of bacteria during antibiotic treatment (third column) or measuring resistance factor adhesion or bacteriophage–bacterium interaction.*

We wrote a review article as well about the effects of natural compounds on cell a dhesion and movement (*Péter et al. Biomedicines, 2021*). Here we systematized the existing knowledge concerning the mechanisms of how natural metabolites affect adhesion and movement, and their role in gene expression as well. We conclude by highlighting the possibilities to screen natural compounds faster and more easily by applying new label-free methods, which also enable a far greater degree of quantification than the conventional methods used hitherto. We have systematically classified recent studies regarding the effects of natural compounds on cellular adhesion and movement, characterizing the active substances according to their organismal origin (plants, animals or fungi). Finally, we also summarized the results of recent studies and experiments on SARS-CoV-2 treatments by natural extracts affecting mainly the adhesion and entry of the virus.

Small molecule natural compounds are gaining popularity in biomedicine due to their easy access to wide structural diversity and their proven health benefits in several case studies. Affinity measurements of small molecules below 100 Da molecular weight in a label-free and automatized manner using small amounts of samples have now become a possibility and reviewed in our work. In our review article (*Peter et al. Heliyon, 2024*) we also highlighted novel label-free setups with excellent time resolution, which is important for kinetic measurements of biomolecules and living cells. We summarized how molecular-scale affinity data can be obtained from the in-depth analysis of cellular kinetic signals. Unlike traditional measurements, label-free biosensors have made such measurements possible, even without isolating specific cellular receptors of interest. Throughout this review, we considered epigallocatechin gallate (EGCG) as an exemplary compound.