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Plenary lectures

ON ORIENTING ELECTROSTATIC AND HYDRODYNAMIC STEERING EFFECTS IN THE KINETICS OF RECEPTOR-LIGAND ASSOCIATION

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The formation of a diffusional encounter complex by receptor and ligand molecules is a preliminary step in many processes taking place in biological systems [1]. Diffusional encounter is thought to influence or limit the rate of these processes. The traditional Smoluchowski theory, which assumes spherical reaction partners with uniformly reactive surfaces, yields the bimolecular diffusional encounter rate constant $k=4\pi(D_A+D_B)(r_A+r_B)$, where D_x and r_x are the translational diffusion coefficient and the radius of the reaction partner X.

The use of the Smoluchowski equation to determine the rate of formation of encounter complexes by biological molecules is limited due to several factors. First, the receptor and ligand molecules encountered in biological systems are usually not spherical. Secondly, the rate of productive diffusional encounter is modulated by orientational requirements for complex formation – i.e. surfaces of the receptor and ligand molecules are not uniformly reactive. Finally, and third, the speed of the encounter complex formation also depends on the long-range interactions between the reaction partners. The importance of two long-range interactions in the encounter processes has been recognized for a long time. These are electrostatic interactions and hydrodynamic interactions.

The electrostatic attraction between a receptor and its ligand can multiply the rate constant of formation of their encounter complex. This has been termed electrostatic steering of the ligand to the receptor binding site [2]. This electrostatic steering can be tested experimentally by changing the ionic strength of the solvent or modifying the reactants leading to the reduction of their electric charge.

Theoretical considerations lead to the conclusion that the existence of hydrodynamic interactions reduces the speed of the formation of encounter complexes, but the experimental study of these effects is not possible as we do not have the tools to regulate the magnitude of hydrodynamic interactions.

An interesting aspect of the influence of electrostatic and hydrodynamic interactions on molecular association arises in situations where the molecules not only must be brought into close proximity, but also must be oriented correctly in space for a successful encounter (e.g., binding or reaction) to occur. The first papers, the authors of which discuss the orienting effects of electrostatic [3,4] and hydrodynamic [5] interactions accelerating the proper orientation of the ligand towards the receptor binding site during the formation of the encounter complex, appeared nearly three decades ago.

Here, I discuss these issues in the light of more recent works. In particular, I am considering the possibility of an experimental demonstration of the orienting influence of hydrodynamic interactions in the association of molecules.

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LASER INTERFEROMETRY SYSTEM FOR BACTERIAL BIOFILM STUDIES

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Optical properties of chemical substances are widely used at present for assays thereof in biological studies. Instrumental optical methods constitute the canon of standard analytical techniques, used in biological sciences. One of the measurement techniques used in physical sciences, with a potential for new applications in biology, is laser interferometry. This method, based on the phenomenon of wave interference, enables quantitative substance assays by means of measurement of the difference between light refractive indexes for the studied and control substances. Moreover, properties of laser-generated radiation, i.e. low beam divergence, narrow spectral band, and a high degree of coherence all have a positive influence on assay sensitivity.

In biological studies, a laser interferometry system constructed at Jan Kochanowski University is used. A beam of monochromatic light is emitted by a He-Ne laser and weakened as a result of passage through the polariser. Next, upon transformation into a coherent 80 mm-diameter plane wave, it falls on the beam splitter cube and is separated into two beams. One of them passes through a membrane system dedicated to biological studies, with temperature control equipment. The other beam is directed by the mirror onto a compensator plate. Because the beam splitter cube is used, both beams interfere. The resulting interference images are recorded by a CCD camera and presented on a graphic screen. A computer image-processing system, complete with dedicated software, enables mathematical analysis of interferograms shown on the system screen. Moreover, taking a series of pictures of interference images in time and conducting a mathematical analysis thereof enables quantitative analysis of real-time substance release kinetics and substance concentration distribution in near-biofilm surface fields. The result of light beam interference when passing through materials with varying refractive indexes is the phase difference between them, which causes a shift of interference stripes. The computer software scans areas within any distance from the biofilm surface with the resolution of the used CCD camera and specifies the deviation of a stripe from its straight-line path at all points. While analyzing their curves, it is possible to determine substance concentration distribution at any distance from the biofilm surface. On the basis of obtained experimental data, the amount of transported substance through the biofilm matrix is calculated.

The laser interferometry system was used to measure the growth medium diffusion through *Pseudomonas aeruginosa* PAO1 biofilm incubated with bacteriophages. This method is able to determine the biofilm matrix-degrading agents activity, as bacteriophages [1,2]. In this method, the biofilm degradation degree is positively correlated with TSB diffusion through the matrix.

Moreover, the laser interferometry system might be used to determine the diffusion properties of biologically active agents transported through bacterial biofilm. [3].

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FLUORESCENCE CORRELATION SPECTROSCOPY STUDIES OF PROPERTIES AND INTERACTIONS OF INTRINSICALLY DISORDERED PROTEINS INVOLVED IN REGULATION OF GENE EXPRESSION

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Gene expression in living organisms is a fundamental cellular process controlled in a complex manner at many different levels. One of them is microRNA-dependent gene silencing by translation repression and mRNA 3' deadenylation. In this silencing pathway, the specific miRNAs bind to the targeted mRNAs and recruit a multiprotein machinery. One of the pivotal components of this huge complex is the glycine and tryptophan rich protein of 182 kDa (GW182) [1] that binds subsequently the scaffolding CNOT1 subunit of 3' deadenylase complex CCR4-NOT [2] through its silencing domain (SD) [3]. While CNOT1 is mostly an α-helical protein, the striking feature of the GW182 SD is the structural intrinsic disorder [4]. The intermolecular interactions of this fuzzy molecular complex [5] are yet not fully characterised.

In this work, we performed fluorescence correlation spectroscopy (FCS) studies of the hydrodynamics and intermolecular interactions of a large set of proteins. We discuss the molecular properties of the intrinsically disordered *vs.* folded proteins in the context of protein dynasome [6], as well as the binding affinity of the key components of the miRNA-dependent gene silencing machinery, *i.e.* different mutants of GW182 SD and CNOT1, compared to protein standards. The results provide biophysical insights into how local structural features of GW182 SD might play a role in regulating its interaction with CNOT1(800-999) fragment.

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MOLECULAR INTERACTION OF SARS-CoV-2 SPIKE PROTEIN WITH HUMAN VIMENTIN

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Vimentin (Vim) is a cytoskeleton protein involved in various cellular functions, including proliferation, cell signaling, maintenance of cellular architecture, and mechanical resistance to external mechanical stress [1-3]. Extracellular Vim has recently been identified as a host cell attachment site for various viral and bacterial pathogens, including SARS-CoV-2 virus [4-6].

This study examined the physicochemical nature of the binding between the SARS-CoV-2 S1 (S1 RBD) glycoprotein receptor binding domain and human Vim. The interaction between S1 RBD and Vim was assessed using AFM-based single molecule force spectroscopy (AFM-SMFS). In order to confirm the experimental results, simulations of the molecular dynamics of Vim-S1 RBD docking were performed. AFM was used to test the interaction between S1 RBD and Vim using two models - Vim molecules immobilized on the mica surface and extracellular Vim present on the surface of a confluent fibroblast culture. In addition to the fibroblasts expressing Vim (mEF +/+), fibroblasts not expressing this protein (mEF -/-) were used as control. The AFM-tips were functionalized with the RBD S1 subunit protein using a heterobifunctional maleimidopropionyl-PEG-NHS linker. A set of force-distance maps for different AFM probe approach/retract rates was collected to investigate the interaction between S1 RBD and Vim. Based on the recorded force curves, the breaking forces between S1 RBD and Vim were calculated. For the AFM approach/retraction speed of 5 μm/s, the mean fracture force of S1 RBD and Vim immobilized on the mica surface was 59 pN. The mean fracture force for the S1 RBD and ACE2 receptor was 33% lower. The bond kinetics was estimated on the basis of the breaking force distribution using Bell-Evans model. The experiment was repeated for the wild S1 RBD strain and 3 variants considered (VOC) and mEF +/+ cells. For an AFM approach/retraction rate

of 5 μm/s, the mean disruption force of wild S1 RBD and Vim cell area was 28 pN. The Vim binding results for the S1 RBD from the UK, Brazil and South African variants were also evaluated. Interactions between Vim and S1 RBD were additionally confirmed using in silico studies.

Our biomechanical studies of the interaction between S1 RBD and Vim provided new evidence of a specific interaction between these molecules. This supports the results of previous biological studies in which Vim was identified as a coreceptor for virus entry.

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REVIEW OF QUANTUM-CASSICAL MOLECULAR DYNAMICS METHODS AND THEIR SELECTED APPLICATIONS IN THE STUDY OF COVALENT DOCKING PROCESSES OF BORATE INHIBITORS

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Quantum-classical molecular dynamics (QM/MM) combines quantum and classical MD algorithms and is able to describe the motions of whole atoms, the electron and/or proton transfer processes in biomolecular structures, as well as the processes of creating and/or breaking chemical bonds. There are two basic problems to be solved: how to generate efficient and reliable potential energy functions, and how to manage dynamics. In practical applications, the division of the configuration space into quantum and classical domains is dictated by the problem under study and the required accuracy of time-dependent solutions. Typically, the dynamics of a quantum subsystem is either described by a stationary, time-independent Schroedinger equation (adiabatic QD) or by an explicit time-dependent Schroedinger equation (nonadiabatic QD), while the rest of the system is usually described by the Newton's time-dependent equations of motion. The coupling between the quantum $\{x\}$ and classical, $\{\vec{R}_\alpha(t)\}$, domains is described by the time-dependent potential function $V=V(x,\{\vec{R}_\alpha(t)\})$ in the Schroedinger equation, and also uses Hellmann-Feynman forces $\vec{F}_\beta = \left\langle \psi \left| \frac{\partial H}{\partial \vec{R}_\beta} \right| \psi \right\rangle$ modifying classical forces in the Newton's equations of motion.

For an overview of the approaches to quantum biology, as well as to a number of QM/MM models and theories, see [1-5]. It should also be emphasized that research into complex contemporary biological problems requires a network of interrelated disciplines, including bioinformatics, quantum computing and machine learning, see the commentary of the global forum on synthetic biology [6].

We have recently developed molecular covalent docking QM/MM protocols to design new classes of inhibitors that form chemical bonds with their biological targets. In particular, the strategy for the design of boron-based inhibitors, holds great promise for enzymes produced, among others, by antibiotic-resistant gram-negative bacteria. We simulated the covalent docking process of inhibitors based on boronic acid and bicyclic boronate scaffolds for β-lactamases belonging to the A, C and D classes. Molecular fragments containing boron can be

transformed from the neutral sp^2 state to the anionic, tetrahedral sp^3 state. Time-dependent QM/MM simulations indicated several significant geometric preferences leading to covalent docking processes - visible on simulation movies [7]. Our simulation methodologies can support the rational design of boron-based covalent inhibitors for many other enzyme systems of clinical relevance.

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TWO-STEP BINDING OF NEOMYCIN TO AN RNA APTAMER

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A synthetic RNA aptamer, forming a riboswitch named N1, regulates gene expression by binding aminoglycoside antibiotics, such as neomycin. Experiments showed that some mutations hinder the riboswitch activity, particularly the A17G mutation reduces the translation regulation efficiency 6-fold [1]. Recently, we elucidated how single-point mutations in the N1 riboswitch affect the internal dynamics of this aptamer and its complexes with aminoglycosides [2]. The dynamical picture conforms with the experimentally determined aminoglycoside dissociation constants [1,3]. In the current study, we focus on the association of neomycin to the N1 riboswitch in order to elucidate the binding process. We apply two-dimensional replica-exchange molecular dynamics to enhance conformational sampling by varying both the temperature and RNA-neomycin distance.

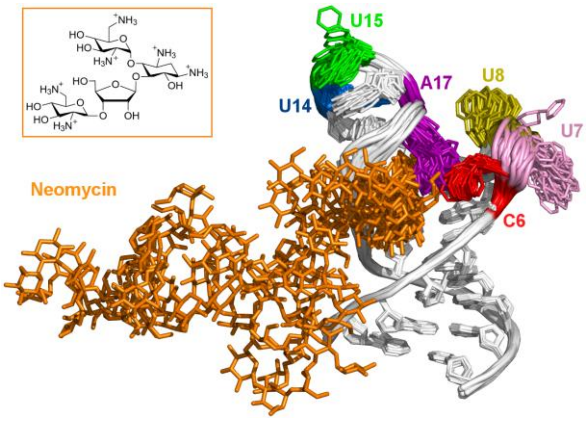

Fig.1. Superposition of replica structures with different RNA-neomycin distances.

The simulations indicate that neomycin binding occurs via one predominant direction even though the shape of the RNA aptamer allows for other binding paths. The two-dimensional free-energy surfaces show two stable low-energy minima along the pathway, suggesting a two-step binding mechanism. The energetically most

favorable minimum corresponds to the neomycin-bound state [2]. The slightly less favorable one likely corresponds to an intermediate state detected experimentally [4]. In the latter local minimum, the G17 nucleobase of the A17G mutant interacts with U7 in a different way than A17 in the N1 riboswitch. We propose that this difference reduces the binding affinity of neomycin to the A17G mutant compared to the wild-type.

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PERMEABILIZATION OF OUTER BACTERIA MEMBRANE BY NANOPARTICLES TO ENHANCE ANTIMICROBIAL EFFECT OF ANTIMICROBIAL PROTEINS-LYSOZYME AND ENDOLYSIN

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Antibacterial resistance has become a global threat, in particular, multidrug-resistant (MDR) bacteria with a broad spectrum of virulence factors. The main problem with bacteria resistance is that they can acquire or develop resistance to commonly used antibiotics. The antibacterial agents currently in clinical development are mostly derivatives of well-established antibiotic and they are affected by pre-existing cross-resistance, which may reduce their efficiency in critical ill patients. Nowadays, several strategies have been studied to overcome multi drug resistance including bacteriophages, antimicrobial proteins (lysins), stimulators of immune system or membrane permeabilizator (eg. antimicrobial peptides and nanoparticles). Among various permeabilizer candidates, cationic nanoparticles are of particular interest because they showed potent antibacterial activity. This may not only lead to bacteria death but also create a route for additional antibacterial compounds eg. phage lysins for peptidoglycan degradation. Bacteriophage-encoded endolysins have emerged as a novel class of antibacterial agents to combat the surging antibiotic resistance. Lysins act as efficient antimicrobials with economical potential. The PG degrading effect of lysins can be seen as osmotic lysis of targeted cell, making these enzymes a desirable and efficient antibacterial agent. However, its use against G-ve bacteria is limited because the outer membrane (OM) of Gve bacteria hinders the permeation of exogenously applied lysins. Therefore, the complexation of nanoparticles with phage-derived endolysin and lysozyme can improve their antibacterial properties against gram-negative bacteria [1-4]. The nanoparticles can be complexed with endolysin or lysozyme, where nanoparticles act as permeabilizers of the bacterial outer membrane (OM) and thus can lead to strengthening bactericidal activity of antimicrobial protein responsible for the degradation of peptidoglycan PG. This helps to create a new tool to fight with multi drug resistance bacteria.

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SUPER-RESOLUTION FLUORESCENCE MICROSCOPY

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New microscopic techniques make it possible to overcome the limitations of traditional optical microscopy. These techniques include: SR-SIM (Super-Resolution Structured Illumination Microscopy), photolocalization techniques: PALM (Photoactivated Localization Microscopy), STORM (Stochastic Optical Reconstruction Microscopy) and others. On the other hand, traditional techniques of fluorescence microscopy can be extended to FLIM (Fluorescence Lifetime Imaging Microscopy) technique.

The presented system is based on a classic wide-field fluorescence microscope, which also enables observations in transmitted light. Most of all, however, what distinguishes this microscope from traditional ones are the modules responsible for super-resolution techniques and fluorescence lifetime imaging.

The SR-SIM and photolocalization techniques use lasers with wavelengths of 405, 488, 561 and 639 nm, and in the FLIM technique - pulsed lasers with wavelengths of 375, 405 and 488 nm. SR-SIM enables observation with a resolution of approx. 100 nm in the XY plane and less than 300 nm in the Z axis, which is two times better than in classic optical microscopes. This improvement in resolution is obtained for standard samples using classic fluorescent markers (Fig. 1). On the other hand, in the PALM technique, the use of special photoconvertible or photoactivatable fluorescent probes allows the observation of objects with sizes well below 100 nm. The TIRF (Total Internal Reflection Fluorescence) technique allows to observe the fluorescence signal coming only from a thin layer with a thickness of 100-200 nm, which removes the blurry background, improving the contrast.

The FLIM technique uses a confocal system mounted in the side port of the microscope and enables point-by-point scanning of the sample, not only to visualize the intensity of the emitted light, but above all to visualize the lifetime of fluorescence and phosphorescence.

In all of the techniques mentioned, 3D imaging is possible, the maximum depth from which information can be gathered depends on the wavelength and the transmittance of the sample. Such a combination of super-resolution and FILM techniques gives the possibility of many unique, previously unavailable observations.

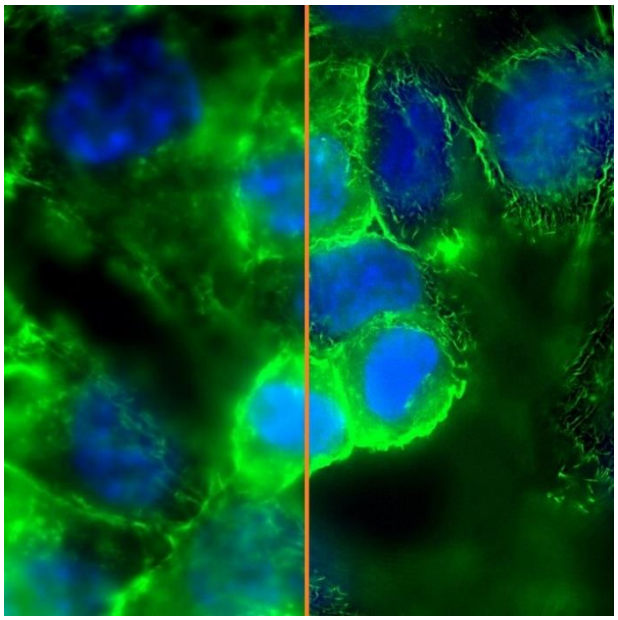

Fig. 1. The image of the tumor cell line obtained by classical fluorescence microscopy (left) and SR-SIM super-resolution microscopy (right). The blue and green colors represent the selectively stained cell structures visible when excited with light at 405 and 488 nm, respectively. The imaged sample area is 125 x 125 μm^2. Source of the sample - Department of Histology and Embryology, CM UMK.

MULTIPLE ASPECTS OF PROTEIN CONFORMATIONAL DYNAMICS REVEALED BY HYDROGEN DEUTERIUM EXCHANGE

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Discoveries of the last years showed that a large portion of the proteome consists of proteins which are not able to form a rigid, well-defined three dimensional structure, constituting the realm of intrinsically disordered proteins/regions (IDP/IDR), the presence of which is widely accepted now. The initial skepticism on their functional role in biology has also diminished due to flow of data on IDRs and their biological activity. Multiple cases of biologically important proteins have been found to contain large IDR's and studied, leading to the inventory of new functional classes that in some cases require disorder. Multiple functional advantages of the dynamics are also mentioned allowing for multivalency, moonlighting, mimicry, plasticity,

dynamic allostery, ultrasensitive signal integration, threshold PTM triggering, fuzzy molecular recognition, entropic chains/bristles, liquid–liquid-demixing phase separations, functional amyloids and alike. New techniques have enriched the portfolio of protein structural studies that enable to tackle the dynamic character of these subjects. The old approach to monitor the kinetics of exchange of backbone amide hydrogens to deuteria (HDX), and therefore to map the entanglement of these hydrogens in secondary or tertiary structure, gained new momentum as an ideal tool to get unique insight into protein regions characterised by different levels of dynamics, the timeframes of which in proteins may span several orders of magnitude. Selected cases from more than 40 protein systems studied by HDX in our lab will be presented and the dynamics-function relationships emerging from these studies will be discussed. These cases include intermediate filament proteins, centriole proteins and translation elongation protein complexes to illustrate the progress in understanding the dynamic aspects of protein function, still not appreciated due to domination of crystallocentric paradigm, as we witness the change in this paradigm and fall of the dogma that function requires structure.

GLUCOCORTICOIDS AND NATURAL KILLER CELLS: A SUPPRESSIVE RELATIONSHIP

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Glucocorticoids exert their pharmacological actions by mimicking and amplifying the function of the endogenous glucocorticoid system's canonical physiological stress response. They affect the immune system at the levels of inflammation and adaptive and innate immunity. These effects are the basis for therapeutic use of glucocorticoids. Innate immunity is the body's first line of defense against disease conditions. It is relatively nonspecific and, among its mediators, natural killer (NK) cells link innate and acquired immunity. NK cell numbers are altered in patients with autoimmune diseases, and research suggests that interactions between glucocorticoids and natural killer cells are critical for successful glucocorticoid therapy. Production and release in the blood of endogenous glucocorticoids are strictly regulated by the hypothalamus-pituitary adrenal axis. A self-regulatory mechanism prevents excessive plasma levels of these hormones. However, exogenous stimuli such as stress, inflammation, infections, cancer, and autoimmune disease can trigger the hypothalamus-pituitary-adrenal axis response and lead to excessive systemic release of glucocorticoids. Thus, stress stimuli, such as sleep deprivation, intense exercise, depression, viral infections, and cancer, can result in release of glucocorticoids and associated immunosuppressant effects. Among these effects are decreases in the numbers and activities of NK cells in inflammatory and autoimmune diseases (e.g., giant cell arteritis, polymyalgia rheumatica, and familial hypogammaglobulinemia).

BIOPHYSICAL HALLMARKS OF TUMOR GROWTH AND INFLAMMATION

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Histopathological examination is a very important stage in the diagnosis of diseases. By means of microscopic techniques, it enables the visualization of morphological changes in tissue structures. Individual cells and their structural assembly into tissues can also be characterized using biophysical techniques to describe their rheological properties, which are "invisible" during histopathological examination, but may be helpful in clinical diagnosis.
Nanoscale changes in the rheological properties of cells / tissues can be observed earlier than microscale histopathological changes [1-7]. Overall, the rheological manifestations of inflamed tissues are less understood than those of neoplastic tissues, while the possibility of differentiating inflammatory processes and carcinogenesis is essential for the development of specific tumor mechano-markers.
In this study, the rheological properties of neoplastic and inflammatory tissues, represented by colon cancer and appendicitis biopsy samples, respectively, were assessed. Collected specimens were tested using a HAAKE Rheostress 6000 rheometer. To determine the rheological characteristics, the elastic modulus (G') and loss modulus (G") as a function of shear strain at different tissue compression states were determined. Additionally, to confirm changes in the mechanical properties of inflamed tissues, examination of biopsy specimens was performed using a NanoWizard 4 BioScience atomic force microscope (AFM), BRUKER JPK. The elastic modulus of the tissues (Young's modulus) was determined from the force-distance curves recorded on the tissues. Force indentation curves,

collected using a silicon nitride cantilever with a 4.5 μm diameter polystyrene bead attached, were analyzed based on the Hertz/Sneddon model. The mechanical characteristics of the tumor or inflamed tissues were determined in comparison to the control normal tissue margins. Properties that distinguish neoplastic or inflamed tissue from healthy tissue have been identified. A comparison of the mechanical properties of the healthy and diseased tissues indicates that the inflamed tissues are softer compared to the healthy ones. The obtained results suggest that changes in the rheological parameters during tissue inflammatory processes, which induced cell infiltration and modifications in the tissue stroma, can be considered as the novel mechano-markers. Rheological examination of tissues involved in pathological processes may enhance standard histopathological diagnosis.

ACKNOWLEDGMENTS

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FROM IDEA TO A PRODUCT FOR MEDICAL DIAGNOSTICS - PCR|ONE AND BACTEROMIC

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Academic research and scientific discoveries create opportunities for breakthrough technologies. I will use the examples of two systems for medical diagnostics developed by the Scope Fluidics group - PCR|ONE offering rapid, fully automated genetic detection of infectious agents, and BacterOMIC offering comprehensive characterisation of antibiotic susceptibility of bacterial pathogens. I will describe the path from the idea to the product and the challenges we faced along the way.

INTRINSIC DISORDER AND PHASE SEPARATION OF THE bHLH-PAS TRANSCRIPTION FACTORS

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The basic helix–loop–helix-Per-ARNT-SIM (bHLH–PAS) proteins represent important class of transcription factors (TFs) which expression is specifically regulated by physiological states and/or environmental signals [1]. Representatives of this family perform a wide spectrum of functions, like Aryl hydrocarbon receptor (AHR) acting as receptor for highly toxic dioxins [2], Clock and Aryl hydrocarbon receptor nuclear translocator-like protein 1 (ARNTL1) regulating circadian rhythms of the organism [3] or Hypoxia inducible factor 1α (Hif-1α) [4], acting as a specific oxygen sensor in cells. Lack of balance in these processes are often linked with the genesis of various diseases, including cancer, stroke, or heart diseases [4]. bHLH-PAS proteins are commonly divided into two classes based on their dimerization pattern, with proteins assigned to class I unable to form homodimers and dependent on the dimerization with class II partners which can also form homodimers [5].
The N-terminal part is structurally conserved and contain bHLH domain responsible for dimerization and DNA binding, followed by PAS domains important for the specificity of DNA binding and signal reception. In contrast to defined domains located within the N-terminal part of bHLH–PAS proteins, their C-termini are highly variable. They usually comprise specific

regions responsible for protein–protein interaction (PPI) known as transcription activation/repression domains (TADs/RPDs) [6,7,8] responsible for the specific modulation of the bHLH–PAS TFs action [9]. Importantly, C-termini of the bHLH-PAS proteins were predicted as intrinsically disordered regions (IDRs) [10]. The experimental verification and characterization of the long C-terminal IDRs of bHLH-PAS representatives was performed by our group for Methoprene tolerant (MET) [11] and germ cell-expressed (GCE) proteins [12]. Both proteins were documented as juvenile hormone (JH) receptors in the model organism *Drosophila melanogaster* [13].

The lack of a defined structure is critical for IDR functionalities in hub proteins enabling different signal pathways crossing [14,15]. IDRs found in bHLH TFs were proposed to contribute directly to the evolution of complex multicellularity [16]. The ability of IDR-containing proteins to form multivalent, weak, and transient interactions enable of proteins to undergo liquid–liquid phase separation (LLPS). Interestingly, although in some cases PPI could lead to LLPS formation, there are also instances where LLPS may prevent protein interactions [17,18,19]. In the context of TFs, it is very interesting to consider the putative role of LLPS in fast cellular responses to external stimuli [20,21]. The ability of protein to undergo the LLPS process may be regulated by a wide spectrum of post-translational modifications (PTMs) and alternative splicing [22]. The extended conformation and low compactness result in IDRs as especially good targets for PTMs and proteolytic degradation, which are typical ways of activity regulation in proteins [23], for example Hypoxia Inducible protein (HIF) [24]. Recently, we discussed the importance of disordered character of bHLH proteins and their propensities to LLPS for functioning as TFs [25].

Neuronal PAS Domain-Containing Protein (NPAS4) belonging to the bHLH-PAS family is one of the immediate early genes (IEGs) that can activate mechanisms related to the first defense against many cellular stresses [26]. Importantly, IEGs are regulated by a specific stimulus with no need for a de novo protein synthesis [27]. NPAS4 has been proposed as a novel therapeutic target for depression and neurodegenerative diseases [28] and as a component of new stroke therapies [29]. Despite discovering in neurons, Npas4 was shown to be multifunctional protein expressed in many types of cells, including β pancreatic [30] and endothelial cells [31]. To date, no structural or biochemical characterization of this protein was performed. In the presentation, we aim to discuss NPAS4 in silico analysis [32] and preliminary results of experimental work in the context of IDR, LLPS and putative ways of this protein functioning as cytoprotective protein.

ACKNOWLEDGMENTS

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EXPRESSION OF PROTEINS CONTROLLING THE MITOCHONDRIAL DYNAMICS IN SH-SY5Y CELLS, IN RESPONSE TO STIMULATION WITH DRUG-MIMETIC COMPOUNDS IN NEURODEGENERATIVE DISEASES

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Neurodegenerative diseases (ND) belong to a group of acquired diseases that are genetically coded and result in the destruction of central and peripheral nervous systems. Recently, it has been suggested that mitochondria (MT) may have a great impact on the development of neurodegenerative diseases. In particular, the imbalance of the fusion/fission events of these organelles is observed [1], favoring the MT division mechanism.

To better understand the mechanism of these processes, we have examined the expression of mitochondrial proteins involved in the fusion/fission dynamics, including DRP1 and its phospho-modifications (S616 and S637), as well as Mfn1 [2]. To perform the experiments, we used 24 h incubated SH-SY5Y cells in Mdivi-1, the mitochondrial fission inhibitor, and a potential drug for neurodegenerative diseases, that is proven to break the MT splitting events [3]. Two concentrations of Mdivi-1 in DMSO were used: 0 and 50 μM. After a 50 μM treatment, discernible morphological changes were observed. Subsequently, we have investigated the expression of fission (DRP1, p-DRP1(S616), p–DRP1(S637) and fusion (Mfn1) proteins to understand how the neuroblastoma cells react to the compound used. The Western blotting with densitometric analysis was then performed on the cytosolic fraction and isolated mitochondria. To normalize the results, β-actin and VDAC proteins were used as the loading control, for the described fractions, respectively.

The acquired images and their saturation statistical analysis strongly suggest that DRP1 and phosphorylated DRP1 (Ser616) protein expression levels in mitochondrial fraction decrease with increasing concentration of Mdivi-1.

At the same time, the mitochondrial p-DRP1 (S637) and Mfn1 and all cytosolic proteins remain unchanged. It implies that Mdivi-1 significantly reduced MT fission by decreasing the recruitment of DRP1 to the outer MT membrane and lowering S616 phosphorylation. However, it has not concurrently enhanced the MT fusion. The morphological changes in neuroblastoma cells, such as the decreased body projections, were also observed. This may be associated with the decreased MT fission and the obstructed translocation of mitochondria to distant cell regions. The obtained results show that Mdivi-1 acts as the potent drug-mimetic compound in restraining the MT divisions. This feature could be utilized in studies of neurodegeneration. Further investigations, using SH-SY5Y cells, differentiated into neuronal phenotype, are needed.

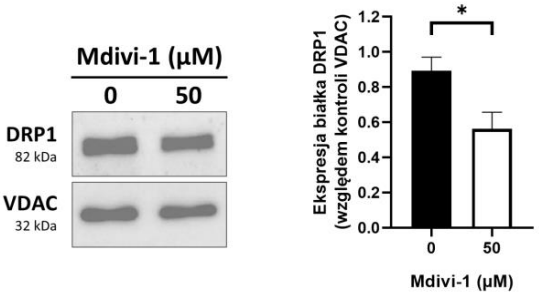

Fig.1. DRP1 protein (82 kDa) expression, relative to VDAC (32 kDa) expression, for 0 and 50 μM Mdivi-1 in MT fraction, using Western blot. Statistical difference marked as * ($p<0.1$).

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ENHANCED FLUORESCENCE DETECTION FOR BIOMEDICAL DIAGNOSTICS AND IMAGING

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Fluorescence-based detection plays a prominent role in modern medical diagnostics and biomedical imaging. Recent progress at the interface of chemistry, physics, micro and nanoscale engineering, nanomaterials, and detectors theoretically enabled ultimate detection sensitivity down to a single molecule level. As single molecule studies in ultra-purified systems and radically limited volume become common the practical biomedical applications are still drastically limited by the background signal that is many orders of magnitude stronger than any single molecule response. Effectively, the broad range of practical applications that include biomarkers detection (e.g. cancer markers or cardiac markers), diagnostics tests (e.g. DNA detection or antibody based assays), imaging and monitoring of intra- and extra-cellular environment to practical forensic applications (like sample collection, processing, and amplification) cannot benefit from single molecule sensitivity.

In this presentation, we will discuss recently developed detection technology that utilizes the difference in fluorescence lifetimes to highly reject background signal and increase detection sensitivity. Taking advantage of longer fluorescence lifetime (10 ns and longer) the excitation pulse sequence highly improves sensitivity for detection, diagnostics, and imaging.

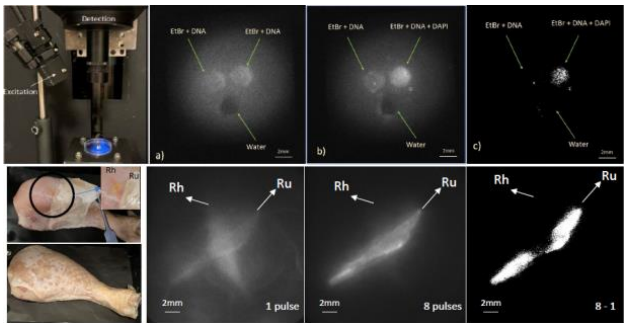

Fig.1. Schematic setup and representative data.

N2-MODIFIED CAP ANALOGUES – A VERY POTENT TOOL FOR mRNA ENGINEERING

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The use of nucleic acids in therapeutics has been intensively researched for many years. The year 2020 proved to be a breakthrough, when the first drug based on mRNA molecule, the COVID-19 vaccine, was introduced to the pharmaceutical market. It was estimated that the COVID-19 vaccine, whose two most commonly used products are formulations containing the mRNA molecule, saved the lives of nearly 20 million people in the first year of the vaccine use.

The mRNA molecule is extremely interesting as a drug. Once developed, the procedure for its synthesis can be applied to obtain many different therapeutic proteins that can be encoded in mRNA sequence. This allows the production process to be quickly adapted to current societal needs.

Intensive research is underway to give mRNA new and even better properties to fully exploit the potential inherent in this molecule. It can be used not only to fight viral diseases, but also in protein supplementation therapy or regenerative medicine[1,2]. One of the components of mRNA that is being intensively studied and modified is the cap structure. This is the element located at the 5' end of the mRNA that provides stability and high translation efficiency. Modification to this region makes mRNA more competitive than native molecules in the patient's cells.

Here, we present biochemical characterization of the new class of RNA capped with newly synthesized dinucleotide cap analogues containing a single aromatic substituent at the N2 position of 7-methylguanosine. We tested the new compounds both alone as inhibitors of the translation process and after incorporation into mRNA. The results showed that the new compounds are 5 to 16 times more potent inhibitors of translation than the standard m^7GpppG cap. These data indicate that the investigated group of analogues has extremely favorable inhibitory properties compared to other compounds studied so far[3].

To characterize the affinity of the newly synthesized compounds for the translation initiation factor eIF4E, we analyzed the thermal stability of murine eIF4E in their presence using DSF. Estimated apparent affinity based on the change in Tm in response to increasing concentrations of the ligand showed that the most potent interacting compounds have approximately 5x higher affinity for eIF4E than m^7GpppG.

We further demonstrated that the new cap analogues are efficiently incorporated into mRNA by RNA polymerases. Interestingly, modifications introduced at N2 guanosine increased the probability of cap incorporation, and for some substituents this modification was sufficient for the analog to be incorporated only in the correct orientation. Thus, the newly synthesized compounds provide an alternative to ARCA-type cap analogues or trinucleotides that ensure correct incorporation into mRNA.

Finally, RNA capped with the newly synthesized compounds showed improved translational properties both in the cell-free translational system of rabbit reticulocytes and in HEK293 cells. In summary, dinucleotide analogs with substituents at the N2 position show much better properties than those commonly used for mRNA preparation, namely m^7GpppG or ARCA.

ACKNOWLEDGMENTS

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LASER EMISSION OF THIOFLAVIN T TO UNCOVER THE EARLY SYMPTOMS OF NEURODEGENERATIVE DISEASES

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Neurodegeneration becomes one of the most commonly diagnosed dysfunction with no certain cure, which has a greater chance of affecting individuals with the age of 65 years or older [1]. Neurodegenerative diseases are commonly associated with neuropathologically distinct amyloid plaques, neurofibrillary tangles and intracellular inclusions in the brain.

Nowadays, the main perpetrators are believed to be small mobile aggregate forms called oligomers, for example misfolded amyloid beta in the case of Alzheimer disease or α-synuclein (α-syn) in the case of Parkinson disease. Oligomers possess the potential to subvert several aggregation pathways and overwhelm cellular functions causing toxicity. Thus it is important to find the way for detecting the toxic oligomer species at the very early stage of their formation, so that patients can receive rapid information about their health condition and have a better outcome in the therapy [2].
A common and widespread method to detect protein aggregates is fluorescence. For that purpose the aggregates are stained with organic molecule named Thioflavin T (ThT) dye which is a gold standard in imaging of neurodegeneration. But fluorescence of ThT lacks the sensitivity to oligomer species.

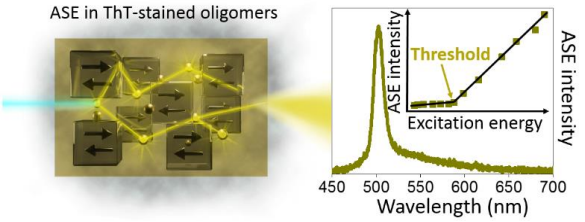

Fig. 1. The amplified spontaneous emission (ASE) of Thioflavin T dye in solid films prepared of early stage protein aggregates. The ASE depends on structural motif, yield of scattering arising from the growing aggregates and seeds of patient's cerebrospinal fluids.

To boost ThT sensitivity fluorescence can be amplified in the process of stimulated emission (Fig. 1). In the amplified spontaneous emission (ASE) process photons emitted spontaneously by excited molecules are multiplied in the stimulated emission process when they interact with other excited molecules during their propagation through the medium (it is the physical mechanism that underlies the operation of lasers). The result is a directional emission of high intensity light with its spectrum significantly narrower than that of fluorescence [3].
ASE was shown to detect aggregation in vitro and in various tissues including the cerebrospinal fluid (CSF), whereby the disease-related protein recombinant is seeded with the patient's fluid. By monitoring the ASE a remarkable recognition sensitivity to pre-fibrillar oligomeric forms can be achieved. Thus, in contrast to fluorescence, ASE, can be used to detect and differentiate amyloid oligomers and evaluate the risk levels of neurodegenerative diseases to potential patients before the clinical symptoms occur [4].

ACKNOWLEDGMENTS

This work was supported by Marie Skłodowska-Curie Fellowship (ref. no: 750917) under Horizon action 2020 and EU Joint Programme - Neurodegeneration Disease Research (JPND) (ref. no: JPND2019-466-136) funded by the National Science Centre, Poland under JPco-fuND 2 granted to P.H.

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ANALYSIS OF FUNCTIONAL DIFFERENCES BETWEEN HUMAN VDAC3 ISOFORMS

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The outer mitochondrial membrane is permeable to ions and small molecules due to the presence of VDAC (voltage-dependent anion-selective channel) proteins. Gene duplication and differentiation during evolution resulted in synthesis of distinct VDAC protein paralogs in almost all organism. In mammals three different VDAC paralogs (VDAC1, VDAC2, and VDAC3) have been identified and among them the biological function of VDAC3 is the least understood [1, 2].
It is known that VDAC3 gene-depleted male mice are infertile due to sperm dysfunction. Moreover two VDAC3 isoforms are known, i.e. canonical and alternative. The alternative VDAC3 isoform results from alternative RNA splicing including additional three-nucleotide (ATG) microexon. Consequently, the VDAC3 isoform contains additional methionine at position 39 [3]. Moreover, the isoform is expressed only in tissues with high energy consumption such as brain, heart and skeletal muscles.
The canonical VDAC3 isoform, but not the alternative

VDAC3 isoform, has been proved to be present in the testes [3]. This suggests that the alternative VDAC3 isoform must play an important role in processes other than reproduction. To explain the alternative VDAC3 isoform function we decided to investigate channel electrophysiological properties and the ability to transport metabolites such as NADH and ADP by the two VDAC3 isoforms.
The results indicate differences in metabolite transport between the two isoforms of the VDAC channel, which may have important implications for clarifying the physiological role of the alternative isoform.

ACKNOWLEDGMENTS

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SEARCH AND PROCESSING OF HOLLIDAY JUNCTIONS WITHIN LONG DNA BY JUNCTION-RESOLVING ENZYMES

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Resolution of four-way Holliday junctions (HJs) is a critical intermediate step of Homologous Recombination. Failure of this process can leave unresolved junctions that covalently link chromosomes together, making cells unable to divide. HJs are resolved by abundant junction-resolving endonucleases that introduce two hydrolytic cleavages. Although their catalytic activity and interaction mode with HJ-DNA have been characterized in great detail by biochemical and structural studies, it remains unclear how the endonucleases find their substrate located in kilobase pairs of duplex DNA.
Here, we studied the interaction of the T7 endonuclease I with a long dsDNA molecule that has a junction located at its center. We employed correlative optical tweezers with confocal fluorescence microscopy to track a single molecule of endonuclease I on the DNA template in real-time. We observed that the enzyme binds remotely to the dsDNA and then undergoes 1D diffusion. Upon encountering the four-way junction, a catalytically impaired endonuclease I mutant remains bound at that point for long periods. If an active enzyme is used, after a few seconds we observe a cleavage event.
We can rationalize all our new data in terms of our earlier crystal structure of T7 endonuclease bound to a DNA junction [1]. We propose that the duplex DNA binds in one of the two DNA binding channels within the dimeric complex. The floor of the channel ha a non-uniform electrostatic potential, and we propose that the DNA duplex will be “floating” in the channel. This model is supported by experiments with an N-terminal 16 amino acid truncation mutant that fails to diffuse on the DNA, indicating the DNA is able to “hover” only because the DNA in the protein channel is encircled by the N-terminal peptide. Such observations would not be possible with any other ensemble or structural approach due to the flexibility of terminal peptides and transient interactions of the “tail-less” endonuclease with DNA duplex.
In this work, we present a robust, quantitative framework to characterize target search by enzymes that recognize DNA secondary structure. Our single-molecule imaging and analysis revealed a comprehensive description of the facilitated diffusion mechanism and the complete resolution trajectory that is likely to be applicable to most junction resolving enzymes.

ACKNOWLEDGMENTS

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MECHANOSENSITIVE CHANNELS - FROM BACTERIA TO MITOCHONDRIA

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All cells sense and respond to mechanical stimuli. Among several molecular machines that react to such cues are mechanosensitive (MS) channels, which are present in the membranes of organisms from all three domains of life: bacteria, archaea, and eukarya. Mechanosensitivity evolved multiple times and MS channels are an extremely heterogeneous group. However, biophysical principles underlying their functioning seem to be common and there are currently two main models of how MS channels are activated. In the first "force-from-lipid" model, MS channels sense directly mechanical deformations of the membrane including changes in tension, thickness, and curvature, and as a result, alter their conformation between closed and open states. In the second model, a spring-like tether links the channel to either the cytoskeleton or extracellular matrix, and displacement of it during stimuli leads to channel opening. Most of the MS channels are still mechanosensitive after reconstitution into pure lipid bilayers supporting the universality of the "force-from-lipid" model.

The physiological roles of MS channels are diverse. Bacteria contain two major classes of MS channels - MscL (mechanosensitive channel of large conductance) and MscS (mechanosensitive channel of small conductance) with typically few MscS paralogs present in a given species. The role of MS channels is insignificant in an osmotically stable setting but is pivotal during osmotic downshocks. Indeed, most bacteria live in osmotically unstable environments, and *Escherichia coli* cells deleted with MS channels do not survive osmotic downshocks. Interestingly, bacterial MS channels have different characteristics including single-channel conductance or the threshold of activation. Some MscS paralogs exhibit intrinsic inactivation (channels close despite constant stimulus) [1]. All this variability seems to be required for fine-tuning cell response to osmotic challenges. In addition to bacteria, MscS-like channels are present also in fungi and plants. A common denominator of these organisms is the cell wall and it was shown that MscS activity might be related to cell wall metabolism in bacteria [2].

Remarkably, one of the MscS-like channels MSL1 in the plant *Arabidopsis thaliana* localizes to mitochondria [3]. Loss of MSL1 resulted in increased mitochondrial membrane potential and a higher oxidation state of the mitochondrial glutathione pool under abiotic stress. This suggests that MS channels might play a regulatory role in mitochondria. MscS-like channels are not present in animals but mitochondrial metabolism seems to be affected by mechanical stimuli in their cells. It might be that other channels exhibiting mechanosensitivity present in their mitochondria like mitoBK$_{Ca}$ [4] fulfill the functions of mechanical sensors. Nevertheless, mitochondrial mechanosensitivity is an uncharted field requiring further exploration.

ACKNOWLEDGMENTS

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KINETIC ASPECTS OF EXTRACELLULAR COPPER TRANSPORT

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Extracellular transport of copper ions and their acquisition by cells is arguably the least understood aspect of copper physiology. There is no consensus as to which molecules carry copper in blood serum and

deliver them to cells, and the knowledge of copper transport in other body fluids is even less advanced [1]. Furthermore, the identified and putative copper transport sites in blood serum proteins and peptides are very inert in exchange reactions, aggravating the enigma. These sites, present in human serum albumin, hCtr1 cellular transporter and some other proteins and peptides belong to the ATCUN/NTS family, characterized by N-terminal Xaa-Zaa-His sequences. They coordinate a Cu(II) ion in a cooperative fashion, saturating its coordination sphere (4N complex, Fig. 1). Previous experiments showed that Cu(II) exchange half-times in these motifs ranges from minutes to days, casting doubt on their physiological role [2,3]. This is because physiological processes have specific timeframes which must be matched by the kinetics of a given chemical reaction to make it biologically useful. Specifically, both the Cu(II) uptake by hCtr1 and Cu(II) release and reuptake in the synaptic cleft apparently occur within a ca. 100 ms time window.

We performed stopped-flow investigations with UV-vis spectral detection on Cu(II) binding to a number of ATCUN/NTS peptides, including simple chemical models, N-termini of identified actors in Cu(II) transport and other relevant biological Cu(II) ligands [4]. The Cu(II) dissociation from some of them was studied using the pH jump approach [5]. In all cases a partially coordinated reaction intermediate was found (2N complex, Fig. 1). It was formed within a millisecond and lasted between ca. 100 ms and several s, depending on a peptide. It was also detected as a dissociation intermediate. Unlike the 4N species, the 2N complex is labile in exchange reactions and can be easily reduced to a Cu(I) species, therefore meeting all criteria for a real physiological Cu(II) transport and exchange species. Our research demonstrated how kinetic studies can reveal actual active species in the biology of metal ions.

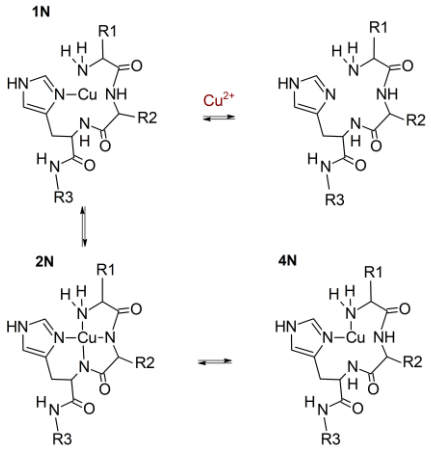

Fig.1. A scheme for Cu(II) binding to ATCUN/NTS peptides. The most reactive conformers for 1N and 2N species are shown [1,4].

ACKNOWLEDGMENTS

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NEW REGULATORY MECHANISMS OF MITOCHONDRIAL POTASSIUM CHANNELS

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Mitochondrial potassium (mitoK) channels play an important role in mitochondrial physiology. Activation of mitoK channels has beneficial effects and can protect brain and cardiac tissue against injury induced by ischemia reperfusion [1]. Mitochondrial, large conductance calcium activated potassium ($mitoBK_{Ca}$) channel was found both in brain and cardiac tissue. Activation of the channel leads to influx of potassium ions into mitochondrial matrix. This induces depolarization of the inner mitochondrial membrane. This simple phenomenon of K^+ influx has multiple consequences for mitochondrial physiology. It was shown that activation of mitoK channels influences oxygen consumption or synthesis of reactive oxygen species in mitochondria [2].

The basic properties of mitochondrial potassium channels, including the $mitoBK_{Ca}$ channel, are very

similar to those of the plasma membrane channels. Interestingly, recent years have shown that these proteins can be specifically regulated due to their location in the mitochondria. Moreover, there are many indications that the activity of mitochondrial potassium channels is regulated by reactive oxygen species synthesized in mitochondria, which is of key importance for the cytoprotection mechanism [3].

The activity of the mitochondrial respiratory chain has been shown to regulate the activity of the mitoBK$_{Ca}$ channel. This suggests a functional and perhaps structural interaction between complexes of respiratory chain and the channel [4]. However, our recent observations put the direct interaction of these proteins into question.

In addition, recent studies indicate the regulation of the mitoBK$_{Ca}$ channel by gasotransmitters such as carbon monoxide or hydrogen sulfide. Electrophysiological studies have shown that the regulation of a channel by carbon monoxide requires the presence of heme bound to the pore-forming subunit of the channel [5].

The presentation will focus on the latest data on the regulation of mitochondrial potassium channels with an emphasis on specific regulation resulting from the localization of these proteins in the mitochondria.

ACKNOWLEDGMENTS

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NANOIR3 - A COMBINED ATOMIC FORCE MICROSCOPE AND NEAR FIELD IR MICROSCOPY (AFM-IR)

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The nanoIR3 is the latest generation system from long history of technology leadership and innovation in AFM-based nano-optical characterization instruments. Designed with an unmatched level of performance, integration, automation, and flexibility, the nanoIR3 sets a new standard for research productivity and ease of use.

The NanoIR3 is a combined Atomic Force Microscope and Near Field IR Microscopy (AFM-IR) system capable of providing nanoscale infrared spectroscopy and chemical imaging, with 10 nm spatial resolution. The system also provides AFM topographic imaging and material property mapping with nanometre-scale resolution [1].

The instrument also integrates an atomic force microscope designed for seamless integration for AFM-IR operation with support for a variety of laser sources. Laser modules allow integration with a user definable number of lasers. It provides nanoscale IR spectroscopy and chemical imaging as well as hyperspectral imaging.

Key Laser Options:

The nanoIR3 supports wide range of lasers for high resolution Tapping AFM-IR spectroscopy and imaging and patented contact mode-based Resonance Enhanced AFM-IR spectroscopy and FASTmapping Imaging. Spectral ranges vary by laser type.

The FASTspectra QCL is a 4 chip QCL laser providing a spectral range of 950-1900cm^{-1}. The laser includes hyperspectral imaging at speeds of approx. <2 second/spectra and FASTspectra spectroscopy of approx. 15 secs for a full spectral sweep.

AFM-IR achieves this goal by using the tip of an atomic force microscope to locally detect thermal expansion of a sample resulting from local absorption of IR radiation. The AFM tip itself thus acts as the IR detector. Because the AFM tip can detect the thermal expansion with spatial resolution approaching the AFM tip radius, the AFM-IR technique can overcome the spatial resolution limits of conventional IR microspectroscopy.

AFM-IR technique has found many diverse applications, including those in materials and life sciences, polymers sciences and technology, including applications on polymer blends, composites, multilayer films, fibers, and conducting polymers. AFM-IR is also finding exciting applications in the life sciences, including subcellular spectroscopy and chemical imaging, nanoscale chemical analysis of tissue, protein secondary structure analysis, including research into protein misfolding related neurogenerative diseases. Additional

applications including photonics, pharmaceutical sciences, perovskites (solar energy), and semiconductors may be also presented.

Total reflection X-ray fluorescence spectroscopy (TXRF) as promising method in analytical chemistry since 1971.

When atoms are irradiated with high energy X-rays, they give off secondary X-rays in the form of fluorescence radiation. The wavelength and energy of the fluorescence radiation is specific for each element, and the intensity of the fluorescence radiation is proportional to the concentration of the each element present in a sample.

In TXRF, the sample is deposited on a reflective disc as a very thin film, and a monochromatic X-ray beam is used to irradiate the sample holder containing the sample at a very small angle (0.3^0–0.6^0) resulting in the total reflection of the X-ray beam. This total reflection reduces the absorption and the scattering of the X-ray beam in the sample and its matrix resulting in greatly reduced background noise, higher sensitivities, and the reduction or elimination of matrix effects [2, 3].

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THE CELL-IN REAGENT AS A METHOD FOR MACROMOLECULES DELIVERY INTO MAMMALIAN CELLS

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Crossing the cell membrane barrier is a critical step in many biochemical and biomedical studies. The great majority of them rely on the use of biomacromolecules that are not spontaneously taken up by the cell due to the selective permeability of the cell membrane. One method of intracellular delivery relies on the disintegration of intracellular vesicles under osmotic shock. During disintegration, probes within the vesicles are released into the cell. This process is used in the new Cell-IN reagent, a product of long-term laboratory research. Cell-IN supports the process of vesicle disintegration - the nanostructure of the working solution proved to be crucial for efficiency.

Cell-IN allows the introduction of a broad spectrum of cargos (dyes, polymers, proteins, nucleic acids, nanoparticles) into different types of mammalian cells (normal, cancer, epithelial, mesenchymal cells). Cell-IN enables the delivery of cargos with sizes ranging from single nanometers to more than 200 nm (diameter).

The effectiveness of cargo delivery for all tested probes and all tested cell lines was assessed quantitatively by measuring the efficiency coefficient, Q (Fig. 1) [1]. Q is the ratio of the cargo concentration inside the tested cells to the cargo concentration in the Cell-IN solution.

High viability (> 80%) of cells undergoing the Cell-IN procedure was confirmed. The percentage of viable cells of the HeLa (cervical cancer) and MDA-MB-231 (triple-negative breast cancer) lines were determined 1h, 1.5h, 2h, and 3 and 4 days after the macromolecules delivery procedure by osmotic shock. Cell viability was examined by two independent cytotoxicity assays: MTT and alamarBlue® (Fig. 2).

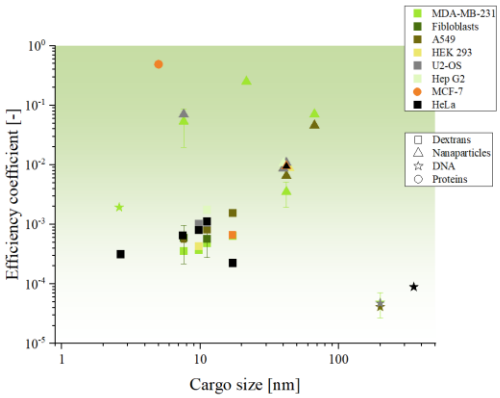

Fig. 1. Efficiency coefficient dependence on a cargo's size, including all tested cell lines and cargo type.

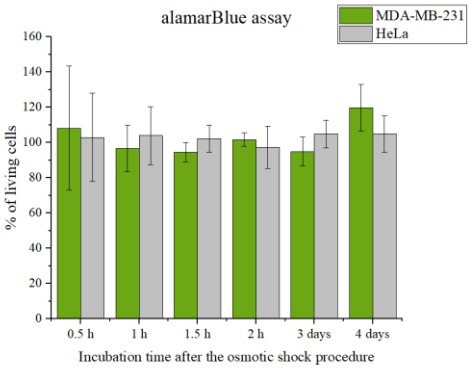

Fig. 2. Comparison of Cell-IN and InfluxTM efficacy for EGFP plasmid delivery into HeLa cells. (A) HeLa cells after EGFP plasmid introduction (24h before imaging) with Cell-IN

product. (B) HeLa cells after EGFP plasmid introduction (24h before imaging) with Influx™ product.

ACKNOWLEDGMENTS

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EFFECT PATERN OF AMINO ACIDS AND NMDA ON ACTION POTENTIALS OF GLR GENES-LACKING MACROALGAE

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Electrical signaling is an integral part of plant physiology. Propagation of action potentials (APs) transduce information to distant parts of the plant body and leads to modulation of vital physiological stimuli [1]. In higher plants, various external stimuli elicit
effect of amino acids and NMDA on the APs of electrical signaling, which a significant role is played by receptor-like homologs of mammalian glutamate receptors (GLR). GLR-mediated responses are calcium-based and have plant-specific physiological functions ranging from carbon/nitrogen metabolism to wound-induced leaf-to-leaf electrical signaling [2]. Despite the wide range of GLR-binding amino acids, classical synthetic NMDA-type iGluR agonist NMDA (binding to the Glu site in the iGluR) was demonstrated to be inactive in higher plants [3]. However, NMDA and amino acids did induce APs in the liverworts – a GLR genes possessing plants [4].

Aquatic Characeanen macroalgae are closely related to land plants, yet they do not passess genes of GLR [5], however, we demonstrated the modulating *Nitellopsis obtusa* – a classical model system of plant electrophysiology [6]. Standardized parameters of action potentials (AP) and excitation current transients were studied in intact internodal cell *via* current clamp and two-electrode voltage clamp modes.

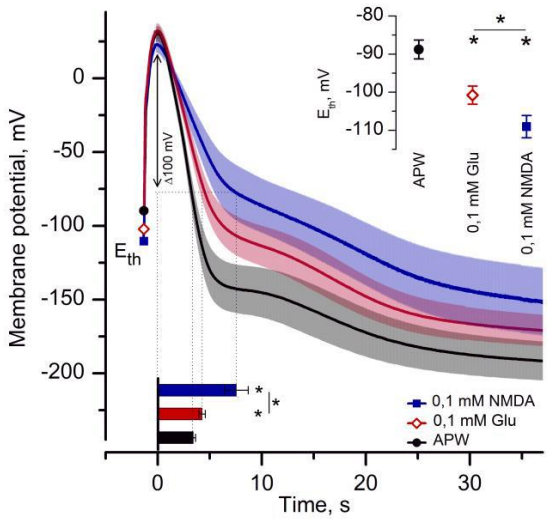

Fig.1. Averaged APs after exposure to 0.1 mM Glu or 0.1 mM NMDA. Solid lines represent mean MP with transparent SE values. Note the differences of AP threshold (E_{th}) and relatively unchanged values of peaks. Dot lines and arrow indicate evaluated repolarization duration (presented in the lower insert). Corresponding E_{th} values are presented in the upper insert. n=8-9.

Results indicate that Glu, Asn and NMDA (0.01-1 mM) increase AP amplitude by hyperpolarizing excitation threshold potential (E_{th}) and prolong AP repolarization phase (Fig. 1). These effects, together with alterations of excitation transient parameters, exhibit dose dependency and a specific effect pattern shared by amino acids and NMDA.Yet, the effect of NMDA exceeds that of Glu. Further, we demonstrated that NMDA-induced alterations are inhibited by ionotropic glutamate receptor inhibitors AP-5 (NMDA-type receptors) and DNQX (AMPA/Kainate-type).

This research presents unpublished correlations between excitation parameters obtained by current-clamp and voltage-clamp approaches and introduces the quantified chloride efflux temporal dynamics during excitation as viable electrophysiological parameters altered by glutamate receptor-like channels' agonists. It finally presents a detailed quantitative description of amino acids and NMDA excitatory effect pattern on APs in a single GLR genes-lacking algal cell.

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EFFECTS OF QUERCETIN AGAINST PARTICULATE MATTER INDUCED IMPAIRMENT OF HBE CELL FUNCTION

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Air pollution and airborne particulate matter (PM) is omnipresent risk factor in modern world. It affects health and it is related to many diseases of respiratory and cardiovascular system. The toxic effects of particulate matter have been linked to many adverse effects within the cell, such as reactive oxygen species (ROS) overproduction leading to e.g. inflammatory response. There is constant search for the new methods reversing the harmful effects of particulate matter induced toxicity.

For more than decade potassium channel openers are known for their protective effects. Moreover it was well documented that mitochondrial large conductance potassium channel (BK_{Ca}) plays crucial role in cytoprotective effect against ischemia-reperfusion events [1].

Cytoprotective and antioxidant effects are well known for many natural components such as flavonoids. However, the exact mode of action is still under investigation. It was previously shown that one of the flavonoids, namely quercetin, can activate mitoBK_{Ca} [2]. Here, we would like to show that quercetin, manifests its fuction in the HBE cells via mitochondrial pathway and mitochondrial BK_{Ca} activation in PM induced toxicity.

The research was conducted on human bronchial epithelial cell line (HBE). The methods incorporated in the study included patch-clamp technique of the inner mitochondrial membrane, transepithelial electrical resistance assessment, mitochondrial respiration measurements with the use oxygen electrode, fluorescence methods for the ROS level and mitochondrial membrane potential assessment, and cell viability measurements using trypan blue staining.

It was observed that PM (<4μm diameter) decreased the transepithelial electrical resistance in HBE cells in dose dependent manner. The effect was partially abolished by quercetin but not by its analog, isorhamnetin. Penitrem A (BK_{Ca} channel inhibitor) reversed the effect of quercetin. The patch-clamp findings confirmed that the effect is associated with channels. Quercetin activated mitoBK_{Ca} channel and the effect was abolished by penitrem A. Isorhamnetin did not affect the channel activity. The results were compatible with mitochondrial membrane and respiration measurements. Quercetin, but not isorhamnetin, decreased the mitochondrial membrane potential and increased mitochondrial respiration. The effect was abolished by penitrem A only in whole cell respiration measurements. Both quercetin and isorhamnetin reveal antioxidant properties. The reduction of PM-induced ROS level occurs both on cellular and mitochondrial level. It correlates with cell viability results for quercetin which increases HBE cell viability after PM administration, whereas isorhamnetin has no effect on cell survival. The toxic effect was also shown on mitochondrial level. The PM incubation with the cells substantially reduced the mitochondrial function measured as respiration control with fully uncoupled mitochondria compared to inhibited electron transport chain. However, neither quercetin nor isorhamnetin could reverse the effect.

The results indicate that PM influences the function of HBE cells on cellular and mitochondrial level. Quercetin is capable to improve the function of HBE cells after PM administration. We show that the effect in HBE cells is connected with its ability to activate mitochondrial BK_{Ca} channel. However, the mechanism of action of quercetin is not exclusively determined by modulation of the channel activity.

ACKNOWLEDGMENTS

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GROUND STATE TAUTOMERIC EQUILIBRIUM AND EXCITED STATE PROTON TRANSFER IN SELECTED PURINE NUCLEOSIDE PHOSPHORYLASE LIGNADS DETERMINED BY QUANTUM CHEMISTRY METHODS

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8-azaGuanine (8AG) and 8-azaisoGuanine (8AIG) are Guanine derivatives in which carbon at position 8 is replaced with nitrogen (Fig. 1). This modification significantly increase intrinsic fluorescence properties of these molecules, which can be utilized for monitoring their interactions with biological polymers like proteins or nucleic acids. In particular these molecules are substrates for ribosylation processes catalyzed by Purine Nucleoside Phosphorylase protein (PNP). In order to better understand protein-ligand interactions it is important to understand the ground-state tautomeric equilibrium as well as possible excited state proton transfer processes, which can take place in these compounds.

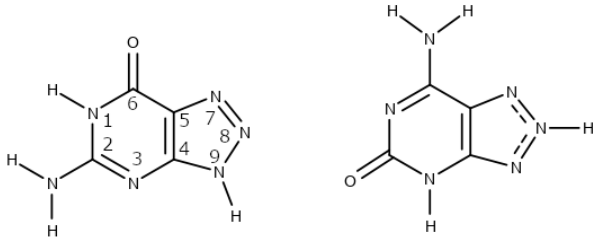

Fig.1. 8-azaGuanine and 8-azaisoGuanine molecules

Ground state tautomeric equilibrium of 8AIG was revealed in our previous publications [1,2]. It was shown that besides the dominant tautomer protonated at positions 3 and 8 some minor tautomers might be present in water solution. Moreover methylation of 8AIG at positions 9 surprisingly significantly increase population of enol forms of the molecule. For 8AG molecule tautomeric equilibrium seems to be much simpler as tautomers protonated at positions 1 and 9 dominates both in water and in the gas phase. Methylation of the molecule at position 9 does not lead to any important shifts in tautomeric equilibrium with dominant form protonated at position 1. The results obtained from population analysis are also supported by vertical absorption energies, which are in a very good agreement with the experimental data.

Calculation of vertical emission energies with TDDFT method sheds light on possible ESPT processes which can take place in both investigated molecules. In particular, vertical emission energies determined for all neutral tautomers of 8AIG strongly support ESPT mechanism proposed by Wierzchowski et al [3]. Therefore, based on the agreement of experimental fluorescence peaks and calculated vertical emission energies, most probable tautomeric species responsible for fluorescence process are suggested for ionic and methylated forms of both molecules.

Moreover, based on our results, we recommend B3LYP and PBE0 functionals combined with aug-cc-pvdz basis set for calculation of vertical absorption and emission energies, respectively for purine analogs.

ACKNOWLEDGMENTS

Most of computations were performed in the Wrocław Centre for Networking and Supercomputing (http://wcss.pl, grant number 349).

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PEPTIDE STAPLING AS A WAY TO ENHANCE ANTIBACTERIAL PROPERTIES OF ANOPLIN

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The emergence of multi-drug resistant bacteria together with the overuse of antibiotics shows an increased need to develop antimicrobial peptides (AMP). One of AMP is anoplin, a linear 10-amino acid peptide extracted from the venom of a soliary wasp [1]. In spite of its weak antimicrobial activity, anoplin has an important property of adopting an amphipathic helix structure near the lipid environment [2]. We thus supposed that stabilizing the active α-helical structure of anoplin would enhance its antimicrobial activity. To test our conjecture,

we introduced hydrocarbon staples into the anoplin sequence.

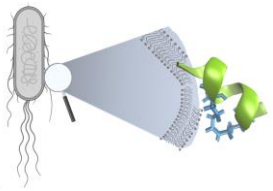

Fig.1. The proposed destructive effect of the stapled α-helical anoplin analog on the cell wall of *E.coli* strain.

Firstly, we designed and synthesized two anoplin derivatives: anoplin[2-6] and anoplin[5-9] by introducing two non-natural amino acids with olefinic side chains into the peptide sequence. The staple insertion sites were chosen so that the charge and hydrophobic-hydrophilic nature of anoplin was unchanged. Secondly, using the Grubbs catalyst, the analogs were covalently linked, i.e., stapled. Thirdly, by circular dichroism spectroscopy, the helical secondary structure was confirmed. The designed stapled peptides exhibited higher antibacterial activity against various bacterial strains, also the resistant ones [3]. Moreover, to investigate if the antibacterial mechanism of analogs is related to their destructive effect on the bacterial cell wall we performed the propidium iodide uptake assay. According to the results, anoplin[2-6] effectively passed through the membrane of *E. coli* K12 so had higher activity against Gram-negative bacteria. However, anoplin[5-9] had a stronger destructive effect on the *S. aureus* Gram-positive cells. Finally, we confirmed that by using the hydrocarbon stapling technique we obtained peptides with stable secondary structures and they did not show toxicity and hemolytic activity [3].
In the future, we plan to conjugate the stapled anoplin to a conventional antibiotic to tackle the problem of bacterial resistance arising due to antibiotic modification.

ACKNOWLEDGMENTS

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RESHAPING MITOCHONDRIAL NETWORK AS A PART OF CELLULAR ADAPTATION IN PRIMARY HUMAN FIBROBLASTS FROM HEALTHY DONORS AND FROM ALZHEIRMER'S DISEASE PATIENTS.

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Mitochondria form a dynamic network, shaped by the processes of fusion and fission, which play an important role in optimizing mitochondrial function. Changes of mitochondrial network morphology impact mitochondrial bioenergetic efficiency and ROS production, they also enable mitochondrial renewal via the processes of biogenesis and mitophagy. Apart from adaptation to changing physiological conditions, alterations in mitochondrial network dynamics accompany various disease states.
We have investigated mitochondrial dynamics in primary fibroblasts derived from patients with Alzheimer's Disease and observed in these cells altered mitochondrial network morphology, as well as a general decrease in the intensity of dynamic processes shaping mitochondrial network: mitochondria fusion, fission, motility and turnover [1,2]. We have also compared the observed changes with the ones characteristics for cellular aging, and with the responses to the physiological insults such as limited nutrient availability [3].

ACKNOWLEDGMENTS

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FEDERATED EUROPEAN GENOME ARCHIVE – POLISH NODE

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The availability of data decreases with the amount of time passed from publication [1]. Such a situation is associated with the "reproducibility crisis" in science [2]. Probably that's why founding organizations like NIH, ERC or NCN, started to require DMPs when submitting grant application. This was also noticed by key players and there were set DbGap [3] by NIH in USA and in Europe - European Genome-phenome Archive [4] by EMBL and Elixir Consortium. Both are online repositories for storing and sharing scientific data on human genetics.

Let's focus on the second imitative since 2013 until 2021 there were sub-mitted more than 12,5 PB of data from about 3 000 studies and more than 30 PB of data were re-used in research. Data from 624 studies were reused at least once. The most re-used data set was used 25 times since publication in the archive. [5]

From 2018, the GDPR came into force in the EU. Member State implementations differed in their approach to sharing human genetic data. To avoid the turbulence associated with the storage of this type of data, the idea of the Federative EGA was born. The idea of distributed archive where data from a given area is stored in a local national node under the jurisdiction of a given country. The Polish node is being created by the University of Lodz based on the government cloud and is intended to be launched fully operationally in the first quarter of 2023.

Free storage and sharing are not the only advantages of FEGA. The functionalities of the repository ensure full control over the deposited datasets thanks to the use of Data Access Committee (DAC) mechanisms and data encryption with the PKI infrastructure.

This tool seems to be the right choice for scientists who want to comply with the requirements of open data policies, DMPs, FAIRness of data, and to increase the chance of theirs work to be seen and reused in international scientific collaborations.

The final argument in favor of a Federated EGA is the savings. When using existing data, costs are significantly lower when compared to a sample acquisition and data generation or even when compared to re-generating data from an already existing sample. With savings in mind, we should not limit ourselves to the economic dimension only. Each time a biological sample is used, its volume is reduced. However, in the case of re-use of existing data, there is no such loss – there appear a saving in biological material.

ACKNOWLEDGMENTS

Founded by Digital Poland Operational Programme Grant Polish Genomic Map in open access - digitization of biomolecular resources of the Biobank Lab POPC.02.03.01-00-0097/19.

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RHEOLOGICAL ASPECT OF BIOPHYSICS OF CIRCULATORY SYSTEM

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Biophysics of circulatory system is an important aspect of medical sciences. Medicine as science of human health and disease treatment and prevention uses results of research within various disciplines. Due to the natural liquid consistence of living organisms, rheology is often applied as a field of science related to the study of liquids, their flow, deformation, and phenomena accompanying this flow. The objects of rheological studies in medicine are primarily blood and body fluids such as joint ooze or saliva, however equally important are the viscoelastic properties of other tissues, like muscles or fat tissue, as well as the micro-viscosity understood as viscous properties in the sub-micron scale, natural for motion inside individual cells and in

the intercellular environment. Rheological tests are also essential for many studies in pharmacy or dentistry. In this work rheological aspects of biophysics of circulatory system are considered

The phenomenon of liquid flow in blood vessels is a very complex process. Blood flow creates friction between both blood components and the blood against the blood vessel wall. According to the Hagen-Poiseuille law, blood flow stream (Q) increases with increasing vessel radius (r) and with increasing pressure difference (Δp) causing flow and decreases with increasing blood viscosity (η) as well as vessel length l (eq. 1) [1,2].

$$Q = \frac{\pi r^4}{8\eta l}\Delta p \qquad (1)$$

Hemorheological studies focus primarily on the measurement of blood viscosity as a function of shear rate, plasma viscosity and red cells aggregability and deformability. Blood, as a suspension of morphological elements in the plasma is a non-Newtonian liquid [1]. Blood viscosity is a strong function of shear rate and it also depends on the sammple history.

Blood viscosity has been studied in many research centers and among many different groups of patients. The main disorders related to the hemorheological properties are: coronary insufficiency, vascular congestion, myocardial infarction, cerebral circulation disorder, Reynaud disease, ischemic limbs, diabetes, anemia, tumors [1-3].

The flow curve measurement involves determining the shear rate dependence of blood viscosity. The viscoelastic properties of blood were investigated in oscillatory measurements. The data obtained from the flow curve measurements can be analyzed using mathematical rheological models like Quemada model in which we use the measured hematoctrite value and plasma viscosity [4].

Blood flow can also be modeled. Numerical fluid mechanics, computational fluid mechanics (CFD) is a tool used to create and solve mathematical models of transfer processes that are based on differential equations of conservation of mass, momentum, and heat [2].

Many hemorheological aspects of living organism functioning are not known yet. We believe that thanks to the new research methods we should learn better the autoregulatory mechanisms aiming at improvement of blood flow. This knowledge could be used to develop alternatives in the case when a living organism cannot do it by itself.

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MECHANICAL UNFOLDING, REFOLDING AND MISFOLDING OF BIOLUMINESCENT PROTEINS: ONE MOLECULE AT A TIME

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Single-molecule force spectroscopy (SMFS)[1] allows individual molecules of biopolymers such as polysaccharides, DNA and proteins to be mechanically stretched into high-energy conformations that are not accessible to other biophysical techniques that operate near equilibrium. Such conformations can be uniquely maintained for a long time and probed. Relaxation pathways to energy minimum structures can be subsequently followed. Using atomic force microscopy (AFM), optical traps (OT) or magnetic tweezers (MT) to execute SMFS measurements it became possible to unravel individual proteins to their extended polypeptide chain conformation and to follow their relaxation pathways until they recover their characteristic 3D fold. Thus, SMFS enabled protein folding study under geometrically restrained conditions that are very different from those using typical bulk biophysical approaches involving thermal or chemical denaturation and renaturation, and arguably closer to the conditions experienced in vivo during the vectorial folding of ribosome-bound nascent polypeptide chains. In this paper, the application of AFM-based SMFS to examine nanomechanics of bioluminescent proteins such as firefly luciferase and Nanoluc is illustrated. Firefly luciferase (Fluc) has been a model, protein for studies of co-translational protein folding as well as a preferred substrate to investigate chaperone assisted refolding, due to its easy bioluminescence readout as a measure of the robustness of its fold. Nanoluc, (Nluc) is an engineered bioluminescent protein that is much smaller than Fluc (170 versus 550 amino acids) yet its light intensity is over 100-fold greater than that

of Fluc. SMFS studies of Fluc revealed that the protein is able to correctly refold to its native structure under partial mechanical unfolding as long as the N terminal domain (~200 amino acids) remains folded. Misfolding occurs when the completely unraveled polypeptide chain is allowed to relax, however Hsp70 chaperones are able to rescue Fluc's fold, apparently, by separating its N and C-terminal domains during AFM-controlled chain refolding. SMFS of Nluc demonstrated that the monomeric protein unfolds and refolds robustly without chaperones' help, however synthetic Nluc constructs composed of tandem repeats of two or three Nluc domains are prone to misfolding under mechanical or thermal denaturation conditions and become chaperones' substrates. The misfolding of Nluc repeats is possibly driven by domain swapping, as suggested by coarse grain molecular dynamics simulations. These observations illustrate unique strengths of SMFS approaches to examine protein folding and misfolding pathways and to probe the mechanism of their interactions with chaperones.

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THE INFLUENCE OF PLANT-DERIVED POLYPHENOLIC COMPOUNDS AND COMPOUNDS FROM THE GROUP OF STATINS AND PHENOTHIAZINES ON DRUG-SENSITIVE AND DRUG-RESISTANT CANCER CELLS AND ON PROPERTIES OF MODEL LIPID MEMBRANES

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Flavonoids, statins and phenothiazines belong to the heterocyclic compounds that exert numerous effects in biological systems. Statins are popular drugs widely used in medical treatment to reduce blood level of cholesterol in hypercholesterolemia. Phenothiazines are commonly used in medicine as tranquillizers. However both groups of the drugs exert also a lot of other biological effects. In many studies on cancer cells various phenothiazine derivatives were shown to act as antitumor, antiproliferative or pro-apoptotic agents. Also spectrum of biological activity of flavonoids which are plant-derived polyphenolic compounds is very broad. The structure of all these compounds enables their specific interactions with different membrane proteins and also non-specific interactions with lipid phase of membranes. Such interactions with membrane components contribute to the various biological effects observed in the presence of phenothiazines, statins and flavonoids.

Phenomenon of multidrug resistance (MDR) is considered to be a major obstacle in successful chemotherapy in tumor diseases. Studies on this phenomenon and its modulation by various groups of compounds are carried out in our laboratory from 25 years [1,2].

Among many different results of studies performed in our laboratory during last years an very important one is that one which shows that combined use of phenothiazines and statins strongly increases accumulation of anticancer drug, doxorubicin in colon cancer cells [3]. It was also observed that flavones such as baicalein and luteolin express anticancer activity [4] and that statins and statins applied in combination with flavonoids reverse drug resistance in colon cancer cells [5].

For better understanding of the mechanism of multidrug resistance and possibility of its overcoming also results of the studies concerning the influence of different group of compounds on properties of lipid membranes are important because lipid bilayer properties control membrane partitioning, transport of P-gp substrates and they may also influence activity of multidrug resistance transporters [6].

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ACTIVATION MECHANISMS OF THE GABA TYPE A RECEPTOR

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GABA type A receptor ($GABA_AR$) is a member of the pentameric ligand gated ion channels family (pLGICs). It plays a crucial role in inhibition in adult mammalian brain and its dysfunction may cause e.g. epilepsy, anxiety and depression. The $GABA_AR$ is composed of five subunits, each consists of extracellular (ECD), transmembrane (TMD) and intracellular domain. Upon the agonist binding at the ECD site, the protein undergoes a series of transitions leading to the opening of the TMD channel gate. Although structures of $GABA_ARs$ are emerging[1,2], the molecular mechanisms of the receptor activation remain elusive. The aim of the presented project was to combine various methods to shed light on these mechanisms.

First, a set of the receptor residues of key importance for the receptor function was selected on the basis of the structure analysis and molecular dynamics simulations. To assess their role, patch-clamp recordings at macroscopic and at single-channel level were conducted for currents mediated by wild type and mutated receptors. These recordings together with modeling enabled us to assess the transition rate constants and to infer the importance of specific residues in distinct conformational changes. In addition, molecular modeling and docking were utilized to further estimate the roles of considered residues.

Our results indicated, that mutations of the residues at the agonist binding site (βE155, βF200, αF64, αF45) are influencing the receptor dose-response relationship to a higher extent than those located in other regions of ECD or TMD (e.g. βF31, αH55, βP273, αL300). On the contrary, the rates of the transitions in the bound states tend to be affected by nearly all mutations, in a manner not clearly dependent on structural localization. This indicates, that the ligand binding is a local phenomenon, but gating has a widespread character. The REFER analysis based on kinetic models was performed to estimate the timeline of structural transitions associated with receptor activation. Two synchronized components of the transition timeline were revealed: the first one composed of movement of the residues located at N-terminal region (βF31, αF14), agonist binding site (βF200, aF64) and ECD/TMD domains interface (βV53, βP273, αH55, αP277) and the second one made of remaining ECD (βE153, αF45) and TMD (βE270, βH267, βL296, αL300) areas. Compared to other pLGIC (AChR)[3], $GABA_AR$ is characterized by relatively high Φ values with a smaller differences in values throughout the protein, suggesting a higher degree of functional compactness of the receptor. Experimental data and molecular modeling underscored the key importance of the interactions at receptor subunit and domain interfaces, providing possible explanation for a relatively low variability in the Φ values in $GABA_ARs$. Thus our data indicated the allosteric character of the receptor activation, characterized by a high level of cooperation and synchronization of the respective protein regions.

ACKNOWLEDGMENTS

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HOW SHEARING AND PULLING AFFECTS GLUTEN PROTEINS: A MOLECULAR DYNAMICS STUDY

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Gluten proteins are responsible for the viscoelastic properties of the wheat dough, and therefore for the bread quality [1,2]. During breadmaking, the dough undergoes various deformations, that change its structure and make it more elastic [2]. In the Institute of Physics, Polish Academy of Sciences we conducted pioneering coarse-grain molecular dynamics simulations of gluten proteins, which allowed us to recreate the effect of shear and pull deformations on gluten proteins. Simulations use implicit solvent, each amino acid is represented by a single pseudo-atom. Details of the model can be found in our articles [3,4]. Periodic

boundary conditions are used in the X and Y directions, but there are attractive solid walls in the Z direction. After preparation and equilibration, the solid walls move periodically in the Z direction (pulling mode) or in the X direction (shearing mode), then after 0, 6 or 10 full oscillations (which are are used to calculate the dynamic Young modulus) they move away from each other in the Z direction, allowing us to calculate the critical strain [4]. During this moving away phase, proteins stick to the opposite walls and rupture into two parts. We measure the force and work needed for this process.

Gluten proteins can be diveded into glutenins and gliadins. The simulations correctly recreate experimental results [2]: glutenins become more resistant to stretching after deformation (see Fig. 1). The control simulations of maize and rice proteins correctly show that they do not become more resistant after being periodically deformed [3]. The resistance rise after deformation is related to a process called strain hardening [2]. We demonstrate that on the molecular level it is based on increasing the number of entanglements and inter-chain contacts. Glutenin proteins are the longest of the proteins mentioned here (the can have over 800 residues), so naturally they can form the highest number of entanglements.

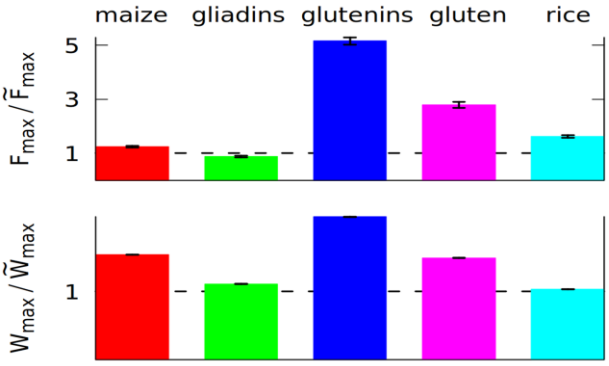

Fig.1. Maximum force and total work required to separate maize, gliadin, glutenin, gluten and rice proteins into two subsystems by pulling the walls in opposite directions. Values are divided by their counterparts measured without oscillations, so the bars show the ratio of values after 6 shear oscillations and after none.

The coordination number measures how well connected is the chain network. It is linked to the Young modulus and critical strain by the slip-bond theory [4]: the more interconnected the network is, the more force you need to rupture it, but the rupture occurs for a lower critical strain (the network breaks all at once), whereas more loosely connected networks are easier to stretch, but can withstand bigger deformations before rupturing. The simulation confirm this for gluten proteins.

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This work would not be possible without Prof. Marek Cieplak, who died recently, and his team at the Institute of Physics, Polish Academy of Sciences.

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DESIGNED RIBOZYME DIRECTED AGAINST ESSENTIAL BACTERIAL MESSENGER RNA

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The spread of antibiotic resistance is becoming a global health threat. An increasing number of bacterial infections and ineffective medicines lead to harder treatment. That problem forces scientific community to develop new efficient antibacterials. A pioneering idea for promising antimicrobial agents lies in ribozymes (RNA enzymes). The catalytic character of natural RNA has been successfully used in, inter alia, anti-viral and anti-cancer therapies [1]. Ribozymes have the capability to bind and digest a specific RNA sequence (Fig. 1). Designing a ribozyme targeting a specific mRNA transcript that encodes a crucial bacterial protein should suppress translation and inhibit microbial growth [2].

We used the hammerhead ribozyme - the best known RNA catalyzing motif. We designed the ribozyme to target the mRNA-*acpP* transcript encoding the acyl carrier protein (ACP) in *Escherichia coli* K-12 MG1655 [3]. The ACP is essential in the fatty acid biosynthesis cycle. Therefore, we expect that preclusion of ACP translation would inhibit bacterial growth.

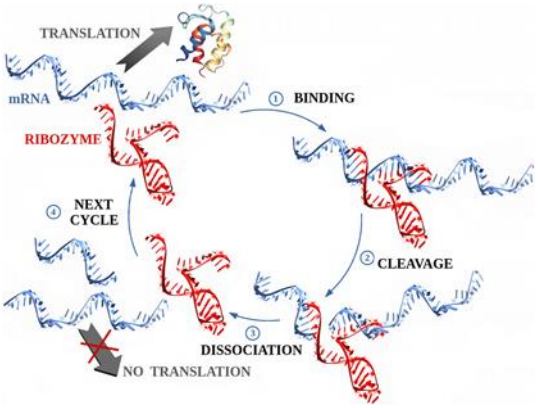

Fig.1. Scheme of cleavage of bacterial mRNA using ribozyme.

We have designed two versions of the anti-*acpP* ribozyme having short or long arms that bind the mRNA. To protect the fragile structure of the RNA enzyme from digestion by endonucleases, we hid the hammerhead ribozyme in a tRNA construct (Fig. 2). This approach should improve the lifetime of the hammerhead ribozyme, and as a consequence, enhance efficiency of cleavage of mRNA-*acpP* in bacterial cells. We have confirmed forming a binding complex of the designed ribozyme to its target in 1:1 ratio by isothermal titration calorimetry. Using gel electrophoresis, we have verified that the mRNA substrate is cleaved effectively *in vitro* by the hammerhead ribozyme. We also found promising results in antibacterial activity of the designed ribozyme.

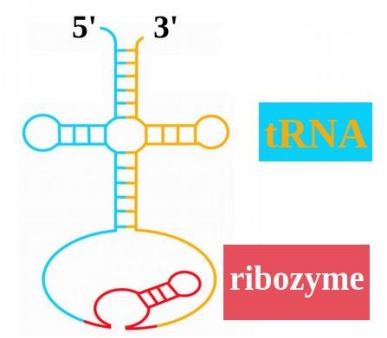

Fig.2. The ribozyme in a tRNA construct.

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ASSOCIATION BETWEEN ALBUMIN AND CHIMERIC LIPID NANOPARTICLES

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Lipid nanoparticles are a well-established class of innovative excipients for the development of drug delivery systems [1]. Lately, advanced platforms that combine multiple types of biomaterials are developed, called “mixed” or “chimeric”, which present new properties and functionalities [2].
Herein, curcumin-loaded lipid/polymer nanoparticles (CLPNs) were developed and their biophysical interactions with human serum albumin (HSA) were assessed.

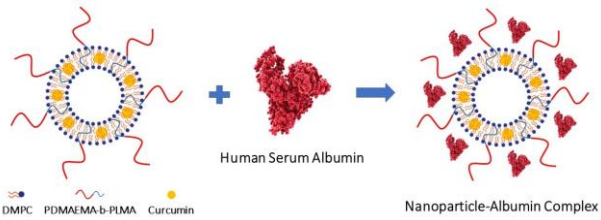

Fig.1. The interaction between HSA and chimeric nanoparticles and the formation of their complex.

Nanoparticles were prepared by thin-film hydration, followed by extrusion, by combining the phospholipid DMPC and the amphiphilic diblock copolymer PDMAEMA-b-PLMA. The particle physicochemical properties were investigated by light scattering, showing small and monodisperse size, positive zeta potential (ZP) and almost complete curcumin incorporation (10% w/w of lipids). In addition, the particles were colloidally stable for at least 15 days.

Table 2. Physicochemical properties of CLPNs.

Property	Value and unit
Hydrodynamic diameter	98.4 ± 1.9 nm
Polydispersity index	0.119 ± 0.016
Zeta potential	14.0 ± 2.1 mV
Incorporation efficiency	99.8% ± 4.1%

The morphology of the chimeric nanoparticles was studied by negative-stain transmission electron microscopy (TEM). The formation of vesicular assemblies of roughly 100nm size was confirmed.

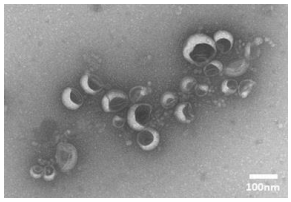

Fig.2. TEM image of the chimeric nanoparticles.

The interactions of CLPNs with HSA were investigated in phosphate buffer (pH = 7.4) by light scattering and circular dichroism (CD). The concentration-dependent effect on the properties of HSA was evident, with saturation occurring at a certain particle concentration in each case. The ZP presented an increase in value, while the system size decreased after the addition of the nanoparticles. Regarding the secondary structure of the protein, a decrease in the a-helical structure was observed, due to a decrease in the band intensity of the CD spectrum at all wavelengths.

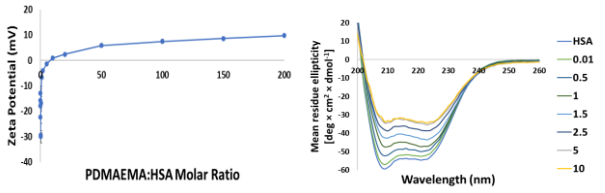

Fig.3. ZP (left) and CD spectra (right) of HSA, titrated with CLPNs at various PDMAEMA:HSA ratios.

Overall, the interactions between the developed nanoparticles (presumably through the cationic PDMAEMA groups) and HSA led to alteration of the surface properties of both elements and to reorganization of the protein domains.
Conclusively, chimeric curcumin-loaded nanoparticles were developed and their association with albumin was established. The study of the biophysical interactions of nanoparticles with physiological components is an essential element for their utilization in biomedical applications.

ACKNOWLEDGMENTS

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PEG AND FICOLL AS MODELS OF CROWDED ENVIRONMENT IN MOLECULAR DYNAMICS SIMULATIONS: TOWARDS UNDERSTANDING DIFFERENTIAL EFFECTS OF SYNTHETIC CROWDERS ON THE HYDROLYTIC ACTIVITY OF A VIRAL PROTEASE

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The highly crowded environment of the cellular cytoplasm is in stark contrast to the conditions of typical biophysical experiments. Volume exclusion and interactions with the crowder molecules can influence the diffusion of enzymes and their substrates as well conformational dynamics and activity of the proteins.
In laboratory experiments, the crowded environment is most often represented with synthetic polymers of various lengths and masses like polyethylene glycol (PEG) and branched polysucrose (Ficoll). However, these molecules are hardly ever used in atomistic molecular dynamics (MD) simulations. Instead, reduced-resolution spherical models or protein crowders are commonly used [1]. This complicates the interpretation of experiments, especially when there are distinct effects of different artificial crowder molecules. Indeed, while we investigated the effects of crowding on the hydrolytic activity of the NS3/4A protease encoded by the hepatitis C virus, we found that the rate of the substrate cleavage was oppositely affected by the presence of PEG and Ficoll [2] . Analysing the trajectories of the NS3/4A protease surrounded with all-atom models of these polymers proved very helpful in understanding the molecular mechanisms behind the effects observed in the experiments. The simulations allowed us to examine protein-crowder interactions with atomistic level of detail, and to assess diffusive

properties of the protein, its substrates and the crowders themselves. This allowed us to develop molecular-level hypotheses for the observed changes in protease activity.

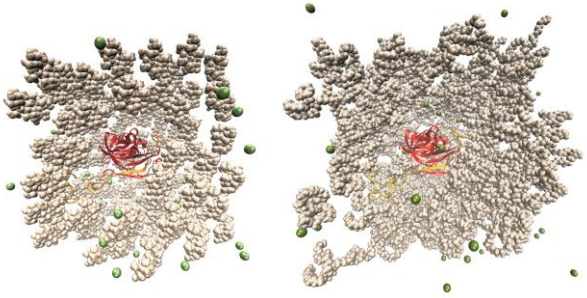

Fig.1. NS3/4A protease from the Hepatitis C Virus surrounded with polysucrose and PEG crowders

The presentation will describe the results of the protease simulations in water and in the presence of crowders, and also cover technical aspects of using non-protein polymers in MD simulations [2,3] . Those include building molecular models of the crowders, assembling the crowded systems, and preventing excessive aggregation in the simulations of a large number of molecules.

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INSIGHTS INTO LIGAND BINDING TO PTERIDINE REDUCTASE 1 FROM MOLECULAR DYNAMICS SIMULATIONS

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Pteridine reductase 1 (PTR1) is a key trypanosomatid-specific enzyme of the folate pathway that is a validated drug target. The roles of the PTR1 dynamics in its function and inhibitor binding are poorly understood. Our preliminary studies indicated that the substrate loop near the PTR1 active site is flexible, which may affect ligand binding [1]. Moreover, crystallographic data suggest that ligand binding may be regulated by the long-distance coupling between four binding sites in the PTR1 homotetramer.

Therefore, we applied molecular dynamics (MD) simulations to characterize the enzyme dynamical properties. The MD results provided insights into the differing interactions of substrates, products and the model inhibitor methotrexate with PTR1. This knowledge may help in designing more effective PTR1 inhibitors. Moreover, simulations with a non-equilibrium method, Rotamerically Induced Perturbations [2], revealed different levels of dynamical coupling between the ligands bound to different pairs of subunits in the PTR1 homotetramer [3], extending our knowledge about the long-range inter-subunit communication in the PTR1 enzyme.

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INTERACTIONS OF PEPTIDE NUCLEIC ACIDS WITH RNA AND BACTERIAL TRANSPORTER PROTEIN BtuB

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Peptide nucleic acid (PNA) is a synthetic DNA analog with a neutral backbone that contains N-(2-aminoethyl)-glycine units instead of the sugar-phosphate ones [1]. A PNA oligomer forms complementary duplexes with itself and natural nucleic acids, and may also form triplexes. The complexes with PNA have higher melting temperatures than those formed by natural nucleic acids [1]. Higher thermal stabilities of short PNA−PNA duplexes over PNA−RNA and RNA−RNA were also confirmed by molecular dynamics simulations at ambient and elevated temperatures [2].

Moreover, PNA is biostable as it is not degraded by neither proteases nor nucleases. Thus, short PNA oligomers are useful in many applications including molecular biology, biotechnology and diagnostics. For example, antisense PNA complementary to mRNA transcripts encoding essential proteins have been useful to inhibit cellular growth, including the growth of bacterial cells (reviewed in [3]).

We have been investigating the interactions of PNA oligomers with bacterial ribosomal RNA and mRNA [2] with the aim to use PNA oligomers as antibacterial compounds. Unfortunately, bacteria do not uptake PNA oligonucleotides and efficient methods for PNA delivery to cells are still being searched for [3]. To deliver PNA to bacterial cytoplasm, we make use of molecules that bacteria need to uptake for growth. Such scarce metabolites enter bacterial cells via energy and receptor-dependent pathway. One of such molecules is vitamin B_{12}. Free vitamin B_{12} enters bacteria via the TonB-dependent transport system and is recognized by the outer-membrane vitamin B_{12}-specific BtuB receptor.

We have previously shown that vitamin B_{12} conjugated to a PNA oligomer, delivers PNA to *E. coli* and *S.* Typhimurium cells [4]. By engineering the *E. coli* $\Delta btuB$ mutant we have confirmed that the BtuB receptor protein is required for uptake of vitamin B_{12}-PNA through the *E. coli* outer membrane [5]. To elucidate atomistic details of this transport we simulated the passage of vitamin B_{12} and the PNA conjugate through the outer-membrane protein BtuB using molecular dynamics simulations. In order to enhance conformational sampling, we developed Gaussian-force simulated annealing method and combined it with umbrella sampling [5,6].

BtuB is a β-barrel protein occluded by a luminal domain. We found that partial unfolding of this domain makes the passage of ligands mechanically feasible [5,6]. PNA movement into the β-barrel is energetically favorable because inside the BtuB protein the hydrophobic PNA extends. In addition, we found that BtuB extracellular loops are actively involved in transport through an induced-fit mechanism.

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NANOPARTICLES AS POSSIBLE ANTICANCER DRUGS ACTIVITY MODULATORS

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Nowadays, the treatment of many cancers is still based on chemotherapy. Commonly used in chemotherapy classical anticancer drugs are often effective against cancer, however, they trigger many adverse side effects. Moreover, many of them cause cell resistance during treatment. The problems associated with the delivery of active drug molecules to cancer cells are another important difficulty in their stable activity against carcinoma.

On the other hand, it is known that many nanoparticles can influence the activity of several antineoplastic drugs. There are many hypotheses that are trying to elucidate this phenomenon. Nanoparticles of various nature may act as efficient drug carriers, directly interact with drug molecules, modify cell receptors and membrane permeability, or even influence various cell metabolism trails.

Fullerene C60 (FC60) is a carbon-based nanoparticle composed of 32 rings. Due to its small size, postulated lack of toxicity, antioxidant activity, ability to non-covalently interact with biomolecules, and capability to penetrate into cells, fullerene became an object of intense research aimed at its application in nanomedicine (drug delivery, protection against free radicals, anti-cancer properties). Fullerene C60 seems to be a great candidate for combination chemotherapy due to its capability to increase the efficiency of anticancer drugs while demonstrating a protective effect on non-cancerous cells [1,2]. Direct interactions of FC60 with various anticancer drug molecules may be one of the most relevant explanations for this phenomenon. Such interactions were examined and demonstrated for FC60 and well-known antineoplastic drugs: doxorubicin and cisplatin [2-5].

Noble metal nanoparticles, like silver- (AgNPs) and platinum nanoparticles (PtNPs), are also interesting candidates for the modulation of anticancer drug activity. It has been shown that AgNPs and PtNPs also can directly interact with antineoplastic drug molecules modulating their biological activity this way [6,7].

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THE NEW MELPHALAN ANALOG (EM–T–MEL) ALTERS THE CONFORMATION OF B-DNA AND INDUCES DNA DAMAGE IN HEMATOLOGICAL MALIGNANCY CELLS

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The second most common hematologic malignancy is multiple myeloma (MM). The treatment program according to a schema (HDM-ASCT) includes high-dose of cytotostatic drug melphalan followed by autologous stem cell transplantation. Melphalan is a bifunctional alkylating agent that covalently binds to nucleophilic sites in the DNA, effective in the treatment but with high systemic toxicity. Therefore, new approaches in drug development are necessary to reduce the side effects, while preserving the high antitumor activity.

Within the scientific framework (Lodz-Warsaw-Erlangen) we synthesized the novel melphalan derivate: [2–(thiomorpholin-methylideneamino)–3–[4–[bis(2–chloroethyl)amino] phenyl] propanoic acid methyl ester hydrochloride] (EM–T–MEL) and analyzed its impact on the DNA in three cancer cell lines: multiple myeloma (RPMI8226), acute monocytic leukemia (THP1) and promyelocytic leukemia (HL60). The ability of EM–T–MEL to affect DNA secondary structure was analyzed by the circular dichroism (CD) technique on native DNA. The level of DNA damage in cells was examined with the alkaline version of comet assay and generation of yH2AX by immunostaining.

We observed the dose-dependent (5-300 μM) changing of the conformation of the B-DNA. Three types of change were seen in the CD spectra: 1) spectral maxima at 275 nm shifted to higher wavelengths, 2) the positive ellipticity decreased between 270 and 290 nm and 3) the negative ellipticity between 233 and 248 nm. At the highest concentration of EM–T–MEL, a peak minimum at λ= 268 nm and maximum at λ= 238 nm were observed. In vitro studies showed that EM–T–MEL causes DNA fragmentation and phosphorylation of the histone yH2AX in all cell lines, and the changes are much higher than in the case of the unmodified drug. The treatment with EM-T-MEL generated DNA damage and phosphorylation of the histone yH2AX time- dependently. After 24 and 48 hours, there was an increased number of cells with abundant foci in the cell and a high percentage of DNA in the comet's tail.

In conclusion, the newly synthesized drug EM-T-MEL modify the conformation of the B-DNA, cause the fragmentation of the DNA and the phosphorylation of the histone yH2AX in a more powerful way than the origin drug.

VISCOELASTICITY OF BRAIN TISSUE AND ITS IMPACT ON GLIOBLASTOMA CELLS

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The brain is one of the the most compliant organs in the human body. Despite its natural softness, brain tissue compression-stiffens and responds in the time-dependent manner under external loads. Single cells within the brain are exposed to forces and stiffnesses higher than those predicted from *ex vivo* tissue measurements. In this regard, it is important to understand how brain cells adopt to an increased stiffness of their surrounding and whether this implies changes in their fate and function. In parallel, brain is also highly viscous, and brain cells' response to viscosity is different from their response to stiffness. Recent development of soft viscoelastic materials where the elastic and viscous moduli can be independently tuned has opened up the possibility to characterize the impact of both elasticity and viscous dissipation on brain cells. The potential of mechanical stimuli to directly influence cell function is relevant to brain tumor growth and essential for understanding how cells and tissues develop under normal conditions and how they change when exposed to altered mechanical loads. Here, the measurements of the brain tissue mechanical properties will be presented and soft viscoelastic materials that mimic brain viscoelasticity will be introduced. The response of the single glioblastoma cells to changes of substrate viscoelasticity will be discussed and that view might shed light on the changes that occur during malignant transformation in brain. Increased mechanical characterization of the brain and further investigations of the mechanobiology of single brain cells under active mechanical forces is needed and have both diagnostic and therapeutic relevance.

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NOVEL BIOSENSING SYSTEMS WITH SELF-QUENCHING FLUORESCENCE NANOFLARES FOR CANCER BIOMARKERS' DETECTION

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Many studies have recently focused on finding new biomarkers, for early detection of debilitating and high-morbidity diseases, that could also serve as the therapeutic targets. Moreover, new methods enabling widespread disease screening while offering a low cost, rapid testing, and biocompatibility, are highly sought after. The studies have shown that biosensing systems based on aptamers and molecular beacons, consisting of single stranded DNA or RNA oligonucleotides in combination with fluorescence detection offer all of these features. Therefore, they can be widely used in many fields, such as the clinical diagnostics, therapeutics, molecular imaging, drug delivery, and biosensing. It has been shown that the Sur protein and its mRNA are highly expressed in most cancers and correlate with resistance to chemotherapy and radiotherapy, associated with increased aggressiveness of tumors. This is making the anti-survivin therapy an attractive cancer treatment strategy. Furthermore, it has been shown that the deviations of ATP concentration from its normal level can be attributed to diseases such as the Parkinson's, cardiovascular disease, carcinogenesis, or cancer progression [1,2]. Therefore, the development of methods that can be used to detect survivin and enable monitoring of ATP concentration level changes are of great significance in studying of the disease severity.

In our first study, we have developed a survivin molecular beacon (SurMB) probe for monitoring Sur mRNA in SW480 cancer cells [3,4]. The probe consisted of a hairpin-like structure with the fluorophore (Joe) and the quencher (Dabcyl). In the absence of the target sequence, the fluorophore and quencher were close together, preventing fluorescence. The presence of Sur mRNA changed the probe conformation, separating the fluorescent dye from the quencher and triggering a fluorescent signal. Moreover, we have demonstrated the ability to detect mismatches in the oligonucleotide sequence and in *in vitro* studies intra-cytoplasmic survivin mRNA in SW480 cells transfected with SurMB probe using graphene oxide and liposomes as nanocarriers.

In our next study, we have developed a fluorescent "ON-OFF" switching probe for monitoring of ATP level changes [5]. For this purpose, a sensor probe consisting of a single-stranded oligonucleotide aptamer, specific for the ATP molecule (Apt (ATP)), was used. In the absence of ATP, a high fluorescent emission signal ("ON") from FAM dye was observed but it was strongly quenched (signal "OFF") in the presence of ATP due to the strong interactions Apt(ATP) with ATP molecule. Furthermore, the selectivity test of the developed aptamer probe was performed over other nucleoside triphosphates CTP, GTP and UTP.

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APPLICATION OF TWO-PHASE MICROFLUIDIC TECHNOLOGY FOR VISCOSITY CHANGE DETECTION IN BIOLOGICAL MICROLABORATORIES

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For various reactions and applications, it has been shown that droplets in microfluidic devices may serve as miniaturized chemical reactors. [1] Such fully operational microreactors can be created in microfluidic systems at rates of up to several kHz, offering fast mixing with an isochronal control of composition reagents. [1,2] Furthermore, large numbers of droplets serving as microreactors can be efficiently processed, observed and analyzed either in series (one after another) or in parallel. [3] The most straightforward chips are linear, i.e., the droplets flow in a single channel. The primary effect governing droplets' motion in such systems is the additional hydrodynamic resistance introduced by each droplet flowing in a microchannel. [4] Therefore, the presence of aquatic droplets suspended in oil changes the hydrodynamic resistance of the network.

Herein, we present how to calculate the additional hydrodynamic resistance caused by a droplet entering into the microchannel. Moreover, since a single droplet can be treated as a bioreactor, we can use it for biological measurements in which the biological process changes the viscosity of the reaction environment.

Firstly, we demonstrate how the method works on the example of culturing bacteria in a droplet for several hours (Fig.1). [5] That case shows the connection between the population size and the formation of bacterial agglomerates versus the bacterial medium's viscosity change. Secondly, we will demonstrate how the difference in the shape of isolated mitochondria can be detected by screening the viscosity of the mitochondrial suspension in a droplet. In turn, that experiment shows how to study the effects of drugs on mitochondrial response expressed by morphological changes.

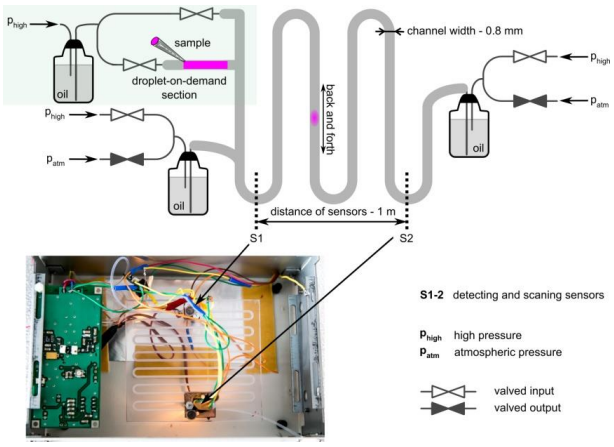

Fig. 1. Schematic of the microfluidic device used in the experiments. The photograph shows the central part of the system with marked sensors.

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ROLE OF DISULFIDE BONDS IN RIBONUCLEASE A

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Ribonucleases are enzymes that catalyze RNA degradation, necessary process for all living organisms. Ribonuclease A from bovine pancreas (RNase A) is the most studied member of ribonucleases and one of the most studied proteins in general[1]. As RNase A needs to form all four disulfide bonds to fold to a native structure, it is used as a model protein for disulfide-bond studies.

Here, we present results of our research, in which we studied formation/disruption of disulfide bonds in RNase A and their influence on protein stability in series of all-atom and coarse-grained molecular dynamics (MD) simulations. The coarse-grained UNRES force field was used to perform simulations, as it is capable of dynamic formation and breaking of disulfide bonds during the course of the simulations, which allows to study influence of disulfide bonds on stabilization of the protein. The reductive/oxidative properties of the environment was controlled by the depth of the disulfide bond formation potential depth. We verified the CG results by running all-atom simulation using AMBER force field with static treatment of disulfide bond and newly-designed pseudo-potential approximating dynamic forming/breaking of disulfide bonds.

Firstly, kinetics and thermodynamics of RNase A unfolding were studied [2] by means of a series of coarse-grained simulation with use of the UNRES force field; namely conventional molecular dynamics (cMD) simulations, run at various temperatures, and multiplexed replica-exchange molecular dynamics (MREMD) [3] simulations in 250-500K temperature range were performed.

Then, additionally, we studied mechanical stability of RNase A using steered molecular dynamics (SMD) by applying additional force to stretch N- and C-termini of the molecule during simulation. Such an approach is a computational equivalent of single-molecule atomic force microscopy (AFM) experiment [4] and allows us to study mechanical properties of the protein in atomistic resolution. SMD simulations were carried out using both all-atom and coarse-grained approaches to allow direct comparison of the data. Due to simplified representation of the polypeptide chains, UNRES force field allows using slower effective pulling speed than in all-atom simulations, which is closer to the one obtained in experimental AFM technique [5]. Both computational and experimental methods are sensitive to the timescale of the experiment as lower pulling speed allows for better equilibration of the system during simulation and may impact the unfolding pathway. Results obtained from SMD simulations were compared with the ones from cMD simulations and available experimental data. [6] This approach allowed us to explain function of each of the disulfide bond and the influence of the redox environment on their behavior.

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PROGRESS IN FLUORESCENT NUCLEOBASE/NUCLEOSIDE ANALOG DEVELOPMENT AND APPLICATIONS

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Recent decade witnessed significant progress in development of fluorescent nucleoside/nucleobase analogs, widely applied in enzymology and cell biology [1, 2]. We were particularly interested in isosteric and/or isomorphic nucleoside analogs, wich usually combine useful spectral properties with biological

activities. Now we briefly review new developments in this field, including those from our laboratory.

Introduction of an "Emissive RNA alphabet", based on thieno-pyrimidine analogs (Figure 1), by Tor and coworkers [3] resulted in many works discussing their rather surprising spectral and enzymatic substrate properties. Recently, this alphabet was extended to involve isothiazole derivatives [4].

Examples of application of this class of compounds to enzyme activity detection and other enzymological research, like RNA depurination, will be given.

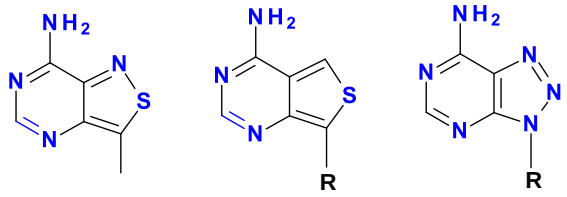

Fig.1. Examples of isosteric nucleoside analogs discussed in this work.

8-azapurines (Figure 1) and their ribosides are another set of fluorescent and isomorphic nucleobase/nucleoside analogs [5]. Their fluorescence was characterized in details in our laboratory, as well as their substrate activities toward several enzymes of purine nucleoside metasbolism. In particular, new non-typical activities of purine-nucleoside phosphorylase towards new analogs, and new methods for quantitation of this enzyme in biological material will be presented.

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NEW INSIGHTS INTO STRUCTURE AND FUNCTION OF PHOTOSYNTHETIC CYTOCHROME B_6F

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Cytochrome b_6f (cytb_6f) is a membrane-embedded homodimeric complex crucial for photosynthetic energy conversion in cyanobacteria, algae and higher plants. Cytb_6f acts on two substrates – membrane soluble plastoquinone and water soluble protein plastocyanin. Reactions of plastoquinone oxidation and plastocyanin reduction catalyzed by cytb_6f provide functional link between photosystem II and photosystem I [1]. We use optical and electron paramagnetic resonance (EPR) spectroscopies to get molecular insights into the operation of cytb_6f.

Overall action of cytb_6f is associated with its structural architecture featuring two redox-active cofactor chains, two catalytic sites (Q_p and Q_n) and cross-membrane electron transfer (ET) fostered by low-spin hemes b_p (hb_p) and b_n (hb_n). This general scheme is shared among all cytochromes *bc*, including bacterial and respiratory cytochromes bc_1 (cytbc_1). A unique feature of cytb_6f is the presence of additional heme (hc_n) at Q_n in proximity to hb_n. The role of this high-spin heme remains elusive. The structural and functional analogy between cytbc_1 and cytb_6f led to a long-standing assumption that relation between midpoint redox potential (E_m) values of hemes *b* in these two enzymes should be the same with E_m of hb_n higher than E_m of hb_p. However, while E_m values of hemes *b* are well established for cytbc_1, uncertainty in determining those values for cytb_6f prevents complete understanding of redox properties of hb_n and hb_p and, subsequently, the energy diagram for the catalytic reaction.

To address this uncertainty, we performed a large-scale equilibrium potentiometric redox titrations of cytb_6f isolated from spinach. All samples were analyzed by cryogenic: i) optical, ii) continuous wave and iii) pulse EPR spectroscopy. Extensive analysis of data obtained for cytb_6f and comparison to data obtained in the same manner for cytbc_1 revealed that in cytb_6f hb_n possesses lower E_m than hb_p. This unexpected result introduces an uphill step in the energy landscape of the enzyme. It follows that the thermodynamic state where both hc_n and hb_n are reduced at the same time is less energetically favorable than state where hc_n and hb_p are reduced. Mechanistic

consequences of this spatial separation of two electrons residing on hemes placed at the opposite sites of the membrane are discussed.

We also reflect on our findings in the context of the structure of plant cytb_6f which provided insights into potential binding mode of plastoquinone at the Q_n site [2]. We expect that a higher-resolution structure with plastoquinones bound at both catalytic sites will further advance our understanding of the mechanism of the catalytic reactions of this enzyme.

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THE INFLUENCE OF CO-APPLICATION OF STATINS AND FLAVONOIDS ON THE ACTIVITY OF VOLTAGE-GATED POTASSIUM CHANNELS kv1.3 AND APOPTOSIS OF Kv1.3 CHANNEL-EXPRESSING CANCER CELLS

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Aim of the study

Influence of co-application of the statins: simvastatin (Sim) and mevastatin (Mev) with each other and with the flavonoids: 8-prenylnaringenin (8-PR), 6 prenylnaringenin (6-PR), xanthohumol (Xanth), acacetin (Ac) and Chrysin (Chr) on the activity of voltage-gated potassium channel Kv1.3 and apoptosis of Kv1.3 channel-expressing cancer T cell line Jurkat. It is postulated that inhibition of these channels expressed in mitochondria (mito Kv1.3 channels) may selectively eliminate Kv1.3 channel-expressing cancer cells, while sparing normal ones. This may open the chance for application of these compounds in a medicinal treatment of some cancer disorders.

Experimental methods

Iinfluence of the compounds on the activity of Kv1.3 channels was examined applying the whole-cell patch-clamp technique. Cell viability upon co-application of the compounds was measured with an application of the MTT Assay. Cell apoptosis was studied by means of measurements of the activity of Caspase-3 and the expression level of Caspase-3 and cleaved Caspase-3, which was estimated applying the Western Blot. Moreover, cell apoptosis was studied by measurements of loss of the mitochondrial membrane potential (MMP).

Results

Co-application of Sim with Mev did not increase the inhibitory effect exerted on Kv1.3 channels upon application of each of the statins alone [1]. On the other hand, co-application of Mev with all the tested flavonoids produced an additive inhibitory effect on the channels. The additive inhibition was also observed upon co-application of Sim with 8-PR, 6-PR and Chr. The most significant inhibition occurred upon co-application of the statins with 8-PR, 6-PR and Chr. Stronger inhibition of the channels occurred upon co-application of Mev with the flavonoids than upon co-application of Sim with these compounds. Application of both statins reduced viability of Jurkat T cells and induced their apoptosis by means of increase of Caspase-3 expression and activity and loss of the MMP. The pro-apoptotic activity was stronger in case of application of Mev than upon application of Sim. Co-application of both statins with all the flavonoids, except for Ac, significantly reduced viability of Jurkat T cells by increasing the pro-apoptotic activity of these compounds. The most significant decrease of the viability, by means of reduction of the concentration of the flavonoid co-applied with the statin, required to diminish the viability to 50% of the control value, occurred upon co-application of Sim with Chr and Mev with 8-PR, Xanth and Chr [1].

Conclusions

1) Co-application of the statins with the flavonoids may produce an additive inhibitory effect on Kv1.3 channels in cancer cells.
2) This effect may be co-related to reduction of viability of Kv1.3 channel-expressing cancer cells due to increase of the pro-apoptotic activity of the compounds.
3) The increased pro-apoptotic activity of the statins co-applied with the flavonoids may be related to inhibition of mito Kv1.3 channels in cancer cells.
4) Obtained results may be important from the point of view of a putative application of inhibitors of Kv1.3

channels to support chemotherapy of cancer disorders, characterized by an up-regulation of Kv1.3 channels, such as melanoma, pancreatic ductal adenocarcinoma or chronic B-type lymphocytic leukemia (B-CLL) [2]. This is because co-application of the statins with the flavonoids may not only improve ant-cancer activity of the compounds, but also reduce potentially required therapeutic dose. This, in turn, may help to avoid unwanted side effects in treated patients due to potential cytotoxicity of the statins [1].

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ENSEMBLE EMPIRICAL MODE DECOMPOSITION OF BK ION CHANNELS SIGNALS

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In the current literature, we can observe some kind of disproportion between the effort put into experimental observations of BK channels and theoretical research. To fully understand the principles underlying these complex microbiological systems, a comprehensive approach, based on both experimental and theoretical results must be implemented.

In this work we decompose the signals which characterize the mitochondrial BK ion channel activity into a finite number of empirical components, using a procedure called *Ensemble Empirical Mode Decomposition (EMD)* pioneered by N. E. Huang et al. [1]. The mode extraction technique allows for a better understanding of the structure of the time series and the complex process hidden behind the data. The EMD technique has been successfully implemented for a wide range of electrophysiological time series. In the case of ionic conductivity signals, this is an innovative approach that allows for the investigation of impact of individual components with different frequency characteristics on the entire signal.

In addition, the received frequency components were carefully analyzed through the methods dedicated to nonlinear and non-stationary signals, including spectral, sample, and multiscale entropy. The investigation of signal complexity employing Entropy measures can ensure a deeper insight into the actual character of the BK channel biosystem [2]. The calculated values of EMD component features were also used for differentiating signals recorded from different types of cell lines (endothelium, fibroblast and hippocampus).

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THE CROSS-CORRELATION-BASED ANALYSIS TO DIGEST THE CONFORMATIONAL DYNAMICS OF THE BK CHANNELS IN TERMS OF THEIR MODULATION BY FLAVONOIDS

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The large conductance voltage- and Ca^{2+}-activated K^+ channels (BK) are regulated by a number of factors. Among them flavonoids, including naringenin (Nar) and quercetin (Que), arise as a promising group of the BK channel modulators from a pharmacological point of view. Although the activating effects of Nar and Que on the BK channel gating are relatively well described in the literature, the appropriate molecular picture of the corresponding channel-ligand interactions remains to be revealed.

In this work, we investigate the functional effects of the Nar and Que on the conformational dynamics of the BK channels. In that aim, we present the results of the cross-correlation-based analysis of the single-channel signals obtained by the patch-clamp method. This allows us

to assess the connectivity and occupancy of distinct conformational substates of the channel in the presence of the considered flavonoids. The obtained results in the form of phase space diagrams allow us to visually monitor the effects of the interactions between the BK channel and Nar or Que at the level of the temporal characteristics of repetitive sequences of channel conformations. The presented results enable us to indicate the ligand-specific effects on channel gating that are related to stronger channel-activation by quercetin than naringenin.

EFFECTS OF STATINS USED IN THE TREATMENT OF HYPERCHOLESTEROLEMIA ON THE OXIDATIVE METABOLISM OF MITOCHONDRIA ISOLATED FROM THE RAT BRAIN

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Atorvastatin and simvastatin are two popular hydrophobic statins that lower cholesterol blood level and are used to treat cardiovascular disease such as hypercholesterolemia. These studies, carried out on the isolated rat brain mitochondria, explain the effects of direct application of these satins on mitochondrial respiratory function, membrane potential and the formation of reactive oxygen species. Both simvastatin and calcium-containing atorvastatin influenced mitochondrial function through increased production of hydrogen peroxide, loss of outer membrane integrity, decrease in maximal respiratory rate, membrane potential, and oxidative phosphorylation efficiency (ADP/O ratio and respiratory control ratio). The action of statins indicates changes in the functioning of the brain's mitochondria by impaired functioning at the level of the respiratory chain, probably in complexes I and III, and at the level of ATP synthesis. The effect of simvastatin appears to be weaker than that of atorvastatin at a given concentration. The stronger effect of atorvastatin on the brain mitochondria was highly dependent on the calcium contained in a given statin and led to the disturbance of mitochondrial calcium homeostasis. The conclusions from this study indicate that hydrophobic statins, widely used as drugs for the treatment of hypercholesterolemia, have a direct negative effect on isolated rat brain mitochondria.

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MONITORING THE MITOCHONDRIAL NETWORK IN SH-SY5Y CELLS UNDER THE INFLUENCE OF POTENTIAL DRUGS IN NEURODEGENERATIVE DISEASES

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The motivation of this work is to explore new effective diagnostic methods in both recognition and therapy of neurodegenerative diseases, as well as to develop a new methodology for studies of drugs with selective activity against Parkinson's and Alzheimer's disorders and utilization of mitochondria as the attractive targets for mitochondrial gene therapy. Mitochondria (MT) are the main intracellular source of crucial biomolecules essential for cellular processes, such as the energy carrier adenosine triphosphate (ATP) and reactive oxygen species (ROS). Mitochondria are also involved in calcium homeostasis and in regulating and initiating cell destructive pathways. The healthy mitochondria form very efficient communicating networks. The morphological abnormalities are indicative of ongoing mitochondrial damage. [1] Therefore, understanding why mitochondrial dysfunction takes a central stage in Parkinson's disease (PD) is integral to combating this debilitating disease. It is the major challenge in developing effective treatments for neurological disorders. Successful therapeutic strategies may halt or slow disease progression instead of merely treating the symptoms.

It has been recognized that the shape and structure of mitochondria can act as functional regulatory factors. At the structural level, MTs are constantly undergoing cycles of fission and fusion. [2] Moreover, alterations in MT dynamics play a crucial part in mitochondrial diseases. [3] Therefore, we anticipate that monitoring MT dynamics (Fig. 1) in response to drugs and disease biomarkers will enable us to understand the role of MTs in various stages of disease development.

Here, we present the development of a system to track changes in the mitochondrial network in SH-SY5Y cells in a microfluidic system, where flow control and drug dosing are carefully controlled (Fig.1). Using Mito Tracker, we show how mitochondrial network dynamics change under the mitochondrial division inhibitor -

Mdivi-1 - a potential drug in neurodegenerative diseases.

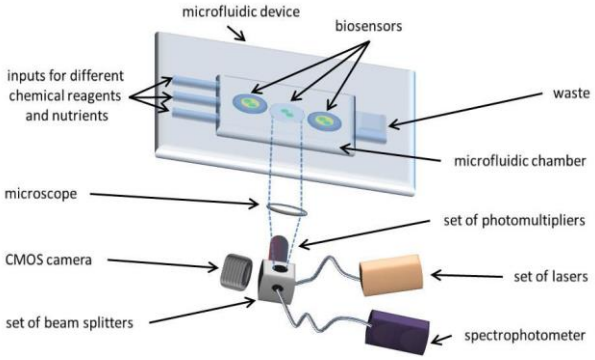

Fig. 1. The scheme of a microfluidic setup with nanogravimetric biosensors to study the response of the cells and mitochondria to chemical stimuli in the microfluidic system (drawn not to scale).

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TIME-RESOLVED FLUORESCENCE SPECTROSCOPY OF BLOOD AND PLASMA IN COVID-19 PATIENTS

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Coronavirus disease 2019 (COVID-19) was proclaimed as a critical global pandemic by the World Health Organization (WHO) in March 2020 caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). There is a need to find new diagnostic and prognostic biomarkers that permit reliable and early diagnosis and risk assessment for mortality and developing persistent pulmonary fibrosis in COVID-19 patients. Oxidative stress-induced by neutrophils and hypoxia in COVID-19 pneumonia leads to albumin oxidation damages. The oxidation protein products trigger oxidative bursts of neutrophils and thus participate in “cytokine storms” to accelerate endothelial lung cell injury leading to respiratory distress and failure. This positive feedback cycle can cause the development of a critical condition or even death in some patients. We suggest that oxidized albumin would be involved in COVID-19 pathophysiology. Some possible clinical consequences of the modification of albumin are also discussed.

We present a new method of oxidative stress assessment on the basis of a small amount of blood sample (5 µl) using time-resolved fluorescence spectroscopy. The findings revealed a remarkable reduction in mean fluorescence lifetime of blood and plasma at 360 nm in patients admitted to the hospital when compared with healthy ($p<0.0001$), further decrease during 1-week hospitalization and a return after 6 months to the level of healthy donors. Moreover, significant negative correlations of mean fluorescence lifetimes with inflammatory parameters (CRP, PCT, WBC), surrogate markers of COVID-19 severity (D-dimer, LDH, troponin, AST), radiological score of computed tomography (HRCT) and positive with albumin were observed. A small amount of blood needed for the tests allows for examining the inflammation from the patient's finger without the burdensome drawing of blood.

Extension of collagen deposition in COVID-19 during injured and scar development in the early phase of wound healing may be associated with the risk of mortality. We found a new method to quickly identify the risk of developing pulmonary fibrosis from a sample of plasma on the basis of fluorescence lifetime measurements at 450 nm.

It can be presumed that fluorescence lifetime may be important in the assessment of the severity of pulmonary inflammation and the risk of death in hospitalized patients with COVID-19. Moreover, it can be used to identify individuals with the post-COVID-19 syndrome. Our results are so far the only ones that investigate the fluorescence lifetime of blood or plasma in patients with COVID-19 and pulmonary fibrosis. Although this hypothesis requires further testing, the numerous benefits of using the fluorescence lifetime measurement, encourage consideration of its application in clinical practice.

This method is non-invasive, low time-consuming, and requires only simple pre-treatment of samples without using additional reagents.

HOW IS INFORMATION DECODED IN DEVELOPMENTAL SYSTEMS?

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The development of multicellular organisms is a dynamic process in which cells divide, rearrange, and interpret molecular signals to adopt specific cell fates. Despite the intrinsic stochasticity of cellular events, the cells identify their position within the tissue with striking precision of one cell diameter in fruit fly or three cell diameters in vertebrate spinal cord. How do cells acquire this positional information? How is this information encoded and how do cells decode it to achieve the observed level of cell fate reproducibility? These are fundamental questions in biology that are still poorly understood.

In this talk, I will combine both information theory methods and mechanistic models to address these questions in the context of spinal cord development [1]. I will consider two opposing morphogen signals that are integrated to specify the arrayed pattern of neural progenitor domains that later on give raise to different type of neurons. Based on the maximum likelihood estimation rule I will define decoding map that provides predictions for shifts in the target gene domains in mutants. The predictions will be validated using experimental data obtained from naïve chick neural plate explants and from embryos with altered ventral morphogen signaling. I will present a simple model of a gene regulatory network that integrates the two morphogen signals and is sufficient to recapitulate the observed shifts in the target domains. I will investigate to what extent the level of noise in the input signals affects precision of the resulting gene expression pattern.

Interestingly the underlying interpretation strategy minimizes patterning errors in response to the joint input of noisy opposing signals. In the long-term, the identified principles of information decoding might be utilized in tissue engineering and neuroregenerative therapies.

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MEASUREMENT OF WATER TRANSPORT ACROSS WILD-TYPE AND CYSTIC FIBROSIS BRONCHIAL EPITHELIUM

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Airways are in the direct contact with the external environment and are acting as a physical barrier between external and internal environment of the human body. Lumen side of airway epithelia are lined by Airway Surface Liquid (ASL) which consists of a low viscosity periciliary liquid (PCL) and the mucus layer (ML). The composition, volume and pH of the ASL are key physiological parameters that are related to airway hydration, reactivity and antimicrobial activity and are precisely regulated [1]. Epithelial ion transport processes regulate the volume and composition of the ASL, mainly through modulation of Na^+ absorption and Cl^- secretion. The crucial role of electrolyte transport in maintaining healthy lungs is illustrated by the lung disease in cystic fibrosis (CF). CF is a life limiting disease caused by mutations in the cystic fibrosis transmembrane conductance regulator (*CFTR*) gene which lead to impairment of ion transport processes and result in abnormal ASL volume and composition [2].

Our previous studies focused of multiple ion transport showed, that the introduction of Na^+ or Cl^- gradients across the epithelium causes the flux of isosmotic NaCl. Introduction of ion transporting proteins blockers present in bronchial epithelium considerably affect the volume of transported fluid [3]. To check the water transport, we designed microfluidic platform that enables accurate measurement of its movements across the epithelium. To test the role of CFTR channel in ASL hydration, we performed the experiments on different cell lines: 16HBE14o- (WT) and CFBE41o- (CF).

Our results show that the water flow through the epithelium exposed to ionic gradients but also in symmetric solutions. The difference between water transport across WT and CF cell monolayers was noticed. Higher water fluxes were seen across WT cells when exposed to Cl^- gradients. The transport rate was higher when low Cl^- solution was introduced on the apical side of monolayer indicating the role of CFTR in this process. In Na^+ gradients, higher water transport in CF cells were seen when high Na^+ solution was introduced on apical side demonstrating the role of ENaC channel in epithelial water absorption.

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ENERGY-DRIVEN PROTEIN DISAGGREGATION: UNSCRAMBLING SCRAMBLED EGGS, ONE POLYPEPTIDE AT A TIME

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Irreversible protein aggregation has been linked to many severe diseases and is a widespread obstacle in biochemical research and biotechnology. ClpB, a heat-shock protein from the AAA+ family of ATPases associated with various cellular activities, possesses a unique capability of unraveling aggregates and reactivating aggregated proteins. ClpB cooperates with the Hsp70/Hsp40 system of molecular chaperones, and this protein-disaggregating machinery is essential for survival of bacteria, fungi, and plants under conditions of severe stress, but is absent in metazoans. A lack of human ClpB makes this chaperone an attractive target for the development of novel antimicrobials.

ClpB, like many AAA+ ATPases, dynamically assembles into ring-shaped oligomers containing either six or seven subunits. The ClpB-mediated aggregate resolubilization is linked to forced extraction of single polypeptides from aggregated particles coupled with their ATP-hydrolysis-driven translocation through a narrow channel at the center of the oligomeric ring of ClpB. Recent cryoEM image reconstructions and single-molecule studies reveal how ClpB utilizes energy from ATP hydrolysis to ratchet substrate polypeptides along the central channel.

Escherichia coli and other bacteria produce two isoforms of ClpB: the full-length ClpB95 and the truncated ClpB80 which does not contain the highly mobile substrate-interacting N-terminal domain. We found that hetero-oligomers of ClpB95 and ClpB80 form preferentially at low protein concentration with a higher affinity than homo-oligomers. Normal-mode analysis of structural oscillations in ClpB showed that the mobility of the N-terminal domain is suppressed in the oligomeric ClpB95 and it is enhanced in the ClpB95/ClpB80 hetero-oligomers. Importantly, hetero-association of ClpB95 and ClpB80 boosts the aggregate-reactivation potential of ClpB, which demonstrates that an oligomeric protein machine can achieve its full activity by assembling from two different types of monomers and underscores how a biological function relies on the modulation of stability and dynamics of an oligomeric assembly.

Within a cellular environment, ClpB must distinguish between properly folded and aggregated proteins by recognizing specific physical and/or chemical surface properties of the aggregates. However, the molecular mechanism of substrate binding to ClpB is poorly understood. We hypothesized that ClpB recognizes those polypeptide segments that promote protein aggregation because they are likely present at the surface of growing aggregates. We used an algorithm TANGO to predict the aggregation-prone segments within the model ClpB-binding peptides and investigated interactions of the FITC-labeled peptides with ClpB using fluorescence anisotropy. We found that ClpB binds the substrate-mimicking peptides with positive cooperativity, which is consistent with an allosteric linkage between substrate binding and ClpB oligomerization. The apparent affinity towards ClpB for peptides displaying different predicted aggregation propensities correlates with the peptide length. However, discrete aggregation-prone segments within the peptides are neither sufficient nor necessary for efficient interaction with ClpB. Our results suggest that the substrate recognition mechanism of ClpB may rely on global surface properties of aggregated proteins rather than on local sequence motifs.

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