Slovak Biophysical Society

Department of Biophysics, Faculty of Science

Center for Interdisciplinary Biosciences,

Technology and Innovation Park

P. J. Šafárik University in Košice

Book of Contributions

8th Slovak Biophysical Symposium

May 30 – June 1, 2018

Košice, Slovakia

Book of Contributions, 8th Slovak Biophysical Symposium, May 30 – June 1, 2018, Košice, Slovakia

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Center for Interdisciplinary Biosciences, Technology and Innovation Park, P. J. Šafárik University in Košice, Slovakia

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Z. BEDNARIKOVA[1], M. GANCAR[1], K. ULICNA[1], 2, D. FEDUNOVA[1], S. S.-S. WANG[3], J. W. WU[4], R. WANG[5], Y. TANG, L. MA[5], B.-B. ZHENG[5], Z. GAZOVA[1]

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[4]*Department of Optometry, Central Taiwan University of Science and Technology, Taichung City,Taiwan*
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S. TOMKOVA[1], V. HUNTOSOVA[2], G. WAGNIERES[3], K. STROFFEKOVA[1]

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J. ULIČNÝ

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E. SEDLÁK[1], A. PLÜCKTHUN[2]

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[2]*Department of Biochemistry, University of Zürich, Zürich, Switzerland*

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E. DUŠEKOVÁ[1], E. SEDLÁK[2]

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M. PETRENČÁKOVÁ[1], D. JANCURA[1,2], E. SEDLÁK[2]

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Institute of Experimental Physics, Slovak Academy of Sciences, Kosice, Slovakia

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Z. KRONEKOVÁ[1], E. PAULOVIČOVÁ[2], L. PAULOVIČOVÁ[2], J. KRONEK[1]

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[2]*Institute of Chemistry, Slovak Academy of Sciences, Bratislava, Slovakia*

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K. KOPČOVÁ[1], L. BLAŠČÁKOVÁ[2], T. KOŽÁR[2], M. FABIAN[2], D. JANCURA[1,2]

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A. ZAHRADNÍKOVÁ[1,2], M. MÁJEKOVÁ[3], J. ŠEVČÍK[4]

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I. ZAHRADNÍK[1], M. HOŤKA[2]

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[2]*Department of Neurophysiology and Neuropharmacology, Center of Physiology and Pharmacology, Medical University of Vienna, Vienna, Austria*

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S. HRIVŇAK[1], J. ULIČNÝ[1], P. VAGOVIČ[2]

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S. HAVADEJ[1]

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Department of Biochemistry, Institute of Chemistry, Faculty of Sciences, P. J. Šafárik University, Košice, Slovakia

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A. ESPINA[1], P. MIŠKOVSKÝ[1,2], S. SÁNCHEZ-CORTÉS[3], Z. JURAŠEKOVÁ[1,2]

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G. FABRICIOVÁ[1], E. LOPEZ-TOBAR[2], S. SANCHEZ CORTES[2], D. JANCURA[1], M. BAČKOR[3]

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L. HUBČÍK[1], J. HAJČIOVÁ[1], S. S. FUNARI[2], D. UHRÍKOVÁ[1]

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S. DATTA[1], A. JUTKOVA[2], P. SRAMKOVA[3], D. CHORVAT[4], P. MISKOVSKY[1,2], D. JANCURA[1,2], J. KRONEK[3]

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N. KANJAKOVÁ[1], T. KONDELA[1], L. HUBČÍK[1], J. C. MARTIÍNEZ[2], D. UHRÍKOVÁ[1]

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K.ŠIPOŠOVÁ[1], E. SEDLÁK[2], M. NEMERGUT[3], T. KOŽÁR[2], A. MUSATOV[1]

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V. VEREBOVÁ[1], K. ŽELONKOVÁ[1,2], B. HOLEČKOVÁ[3], J. DIANOVSKÝ[3], J. STANIČOVÁ[1,4]
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K. ŽELINSKÁ, Z. ZBOŇÁKOVÁ, S. HULÁKOVÁ, J. GALLOVÁ
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PLENARY LECTURES

Single-molecule nanomechanics of protein machines

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Three-dimensional structures of proteins are a source of fascination for scientists, due to the beauty of their sequence-encoded architectures and their highly diverse range of functions. When looking at protein structures at atomic resolution, it is tempting to use macroscopic mechanical analogies to describe their function as molecular machines. However, such analogies are often misleading because boundaries between independently stable subdomains cannot often be determined from structures, owing to the high cooperativity of protein folding and structural transitions. Single-molecule protein nanomechanics have emerged as a tool to force biomolecules through their conformational space and hence identify hinges, breaking points, as well as mechanically stable subdomains [1].

A prominent example of a protein machine undergoing large conformational change during its functional cycle is the ATP-regulated Hsp70 chaperone DnaK – a central molecular chaperone of the protein quality control network in a cell [2]. Once ATP is bound to the nucleotide binding domain (NBD) of DnaK, the initially closed substrate binding domain (SBD) opens its binding cleft by engaging the α-subdomain to the NBD. In doing so, it undergoes a dramatic ∼10 Å displacement of its lid subdomain to allow the exchange of substrates [3]. Several crystal structures of the isolated SBD (in which the NBD is absent) have been solved [4, 5]. In these structures, the absence or presence of peptide clients or non-natural ligands induce no significant structural changes in the closed conformation. There is no indication in the crystal structures of the huge conformational change of the lid domain of the SBD, seen in the ATP-form of the full-length two-domain DnaK. Therefore, the large conformational change of the SBD is only observed in the two-domain DnaK after ATP binding. Thus, even though the crystal structures provide us with very valuable insights into the three-dimensional arrangement of individual atoms, the thermodynamic and mechanical stability of individual substructures are difficult to predict based on this information alone. Here we focused on how the large ATP-induced changes of the SBD, as seen in the two-domain DnaK, are mirrored in the subdomain integrity and nanomechanics of the closed form of the SBD and how this integrity is affected by peptide (client) binding.

Generally, a folded protein which undergoes large conformational changes (such as the substrate binding domain (SBD) of the Hsp70 chaperone) faces a seemingly contradictory demand; a high folding stability and cooperativity ensures that a protein is in a functional state, but at the same time it increases the energy penalty for disrupting the interactions in the native state during conformational transition. Recently, using single-molecule force spectroscopy, we investigated how such conundrum is solved for the SBD of Hsp70 [6]. Specifically, the parts involved in the conformational changes are mechanically weak and therefore amenable to deformations. Such mechanically weak regions are decoupled from the overall protein stability by selective destabilizing the elements of the secondary structures and thus are the Achilles' heels of the protein. In the case of the SBD, β7-8 as well as subdomain interface are elements, which are energy-decoupled from the rest of the protein structure. Interestingly, previously various parts of the SBD have been implicated in a hinge function, based on local structural heterogeneity with a possible impact on allosteric signaling pathways in DnaK [4]. While such thermally accessible states can be readily populated on fast timescales, in our approach, force is used to drive the closed SBD structure toward the open state by providing a large energy bias to investigate collective dynamics at much slower timescales.

Large allosteric shape changes of proteins, including the action of molecular motors as a prominent example, are often discussed using energy landscape concepts which distinguish between lever-arm type active motions of the protein and passive so-called Brownian ratchet models. We propose to apply such a picture also to the process of peptide-induced lid closing. In the case of the SBD of DnaK, an active motion would imply that peptide binding actively drives the lid from an open into a closed conformation through rotation of the hinge elements ("lever arm") (i.e., β7-8). A ratchet model, however, would imply that peptide binding locks the interface between lid and core as soon as they form physical contact but does not drive the elements β7-8 involved in the transition actively. Our results favor a ratchet-type closing of the lid because β7-8 docking energies are independent of bound substrate, and hence substrates do not drive active docking elements. It means that thermal motions agitate the SBD until the lid touches the β-subdomain and the interface energy locks it in the closed conformation (the ratchet mechanism).

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Effect of natural and synthetic small molecules on aggregation of globular proteins

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Amyloid diseases are characterized by the formation and deposition of amyloid aggregates inside or outside the cell. Amyloid-associated human diseases include Alzheimer's disease, Parkinson's disease, prion diseases and type II diabetes. Currently, these diseases are incurable; thus, comprehensive search for novel inhibitors is required to develop treatment strategies [1]. Several types of potential inhibitors among them nanoparticles, short peptides, antibodies and small organic molecules have been studied so far but in spite of intense research, the mechanism of inhibition is poorly understood. However, several drug molecules are proven to be effective against amyloid fibrillization process and have potential to either prevent the aggregate formation or to some extent reverse the process of protein aggregation [2].

Herein we opted to test several small compounds with different structure, either extracted from herbs or obtained from multi-step synthesis, for anti-amyloid properties towards globular proteins. Hen egg white (HEW) lysozyme and human insulin were picked as model systems. The ability of extracts from traditional Chinese herbs (DB series) and synthesized tacrine – coumarin derivatives (SH series) to inhibit amyloid fibril formation of globular proteins were studied using Thioflavin T fluorescence assay, atomic force microscopy and molecular docking methods. The inhibitory activities were further quantified through the IC_{50} values.

The obtained data suggest that inhibitory effect of compounds on lysozyme or insulin fibrillization depends on their composition, not origin as each of three studied series contained compound with weak, mild or strong inhibitory activity. Based on the results we can suggest that important factor is the planarity of the molecule. Extracts from herbs, DB series, were non planar and possess only weak inhibitory activity with IC_{50} values 100-times higher than concentration of protein. On the other hand, the most potent tacrine-coumarin derivatives were observed to influence the protein aggregation at the stoichiometric concentration (IC_{50} ~ 19-100 μM). The functional groups and types as well as length of linker also play key roles in inhibiting the activity of compounds.

Our results provide useful information about the structure and composition for design of novel and potential lead compounds for a treatment of amyloid-related diseases.

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Photobiomodulation affects mitochondria morphogenesis and function

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The world's population is gradually aging. According to the World Health Organization (WHO), between 2015 and 2050, the proportion of the world's population over 60 years will nearly double from 12% to 22% [2]. The aging leads to an increased cardiovascular and neurodegenerative diseases incidence. Increasing number of studies show that impaired mitochondrial function and dynamics play crucial roles in aging and pathogenesis of neurodegenerative diseases such as Parkinson (PD), Alzheimer (AD), and Huntington disease (HD)[3]. Treatments for most neurodegenerative disorders and for neurological trauma focus on slowing down the progress of disease and to alleviate the symptoms. Therefore any additional neuroprotective and neuroregenerative approaches to the classical ones will have great benefit in treating neurological disorders, in particular in terms of socio-economic impact.

Photobiomodulation (PBM) represents very promising therapeutic approach that can improve mitochondria functionality. PBM uses non-ionizing light sources, including lasers and LEDs (light emitting diods), the most often with wavelength (~600–950 nm) in visible and NiR spectrum. PBM effects involve nonthermal processes with endogenous chromophores eliciting photophysical and photochemical events at various biological scales. In the clinical practice, the PBM beneficial therapeutic outcomes include alleviation of pain or inflammation, immunomodulation, and promotion of wound healing and soft tissue regeneration[4,5].

In last decade, mounting evidence appears to the beneficial effects of PBM in treatment of neurodegenerative diseases [6-11]. However, PBM has not been widely adopted in clinical practice for a number of reasons. Therapeutic PBM effects are affected by many factors and conditions such as a spatial distribution of irradiation, treatment timing and repetition, irradiation type (continual or pulse) and wavelength. The implications of light penetration have not been thoroughly addressed, and PBM mechanisms of action are complex at various levels. There are effects at the direct cellular level, in many molecular and cellular signaling pathways, and at the systemic level, where NiR affects neurotrophic factors and synaptogenesis [5, 10,11]. The underlying cellular and molecular mechanisms responsible for the therapeutic value of PBM are not completely understood, particularly with respect to tissue in nerve system.

In our study, we have focused on the acute PBM effects at the direct cellular level in control and rotenone challenged cells (PD model) with respect on mitochondria morphogenesis, cell viability and metabolism. Our results indicate that PBM effects depend on irradiation wavelength and dose. In control and rotenone challenged cells, PBM treatment resulted in significant increase in mitochondria fusion with corresponding improvement in mitochondria function.

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Architecture of cell DNA - X-ray imaging approach

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The structural dynamics of higher eukaryote genomes become lately subject of increased interest, fueled mainly by new experimental information provided by High-C and related experiments [1]. In spite of ongoing efforts, the actual 3D information about the way chromatin is folded in space remains an elusive goal. While topological contact maps provide invaluable information about an active part of genome and corroborate the importance of knowing the actual 3D conformation of chromatin in various cell states [2], the achievable resolution of single-cell experiments leaves a lot to desire.

There are several lines of attack to achieve the resolution necessary to not only understand principal mechanisms and fundamental features of gene expression control and epigenomic programming of the cell, but also to get comprehensive view on the whole process. The highest resolution of High-C class of experiments is at the level of 10 kbp (smallest fragments of sequence), done at statistical ensembles of synchronized cells and subject of probabilistic interpretation and possible preparation artefacts. Cryo-EM, in spite of long years effort struggles with sample preparation issues [ref. Private comm NTU]. Amongst the most promising approach for single-cell imaging is the use of optical super resolution techniques [3,4], but carrying on its own methodological limitations.

We will present here an alternative approaches based on modern coherent X-ray imaging techniques, available on synchrotrons, free-electron lasers such as European XFEL and in the future even on „laboratory“ sources. Our experiments, using coherent diffraction imaging using hard X-ray at ESRF ID10, showed [6], that for existing experimental setup, 3D imaging of sufficiently contrasted sample is possible with spatial resolution at least 27 nm, technically the setup allows even sub-10 nm resolution. Limitations of resolving power are given by radiation damage of fragile biological samples rather than formal resolution limits [5]. While the resolving power seems to be competitive to immunomarkers used for optical super resolution techniques, the ultimate resolution requires careful sample preparation, efficient sequence-specific contrasting techniques and, last but not least, intense use of *a priori* information.

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SHORT COMMUNICATIONS

Analysis of kinetic stability of antibodies by microcalorimetry

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The stability of Immunoglobulin G (IgG) affects production, storage and usability, especially in the clinic. The complex thermal and isothermal transitions of IgGs, especially their irreversibilities, pose a challenge to the proper determination of parameters describing their thermodynamic and kinetic stability. Accelerated stability studies, e.g., by employing thermal denaturation, have the great potential for enabling high-throughput screening campaigns to find optimal molecular variants and formulations in a short time. Surprisingly, no validated quantitative analysis of these accelerated studies has been performed yet, which clearly limits their application for predicting IgG stability.

Here, we present a reliable mathematical model to study the irreversible thermal denaturations of antibody variants [1], validation of the model [2], and its utilization in formulation development [3].

We show that the proper analysis of DSC scans for full-length IgGs and their corresponding Fab fragments not only helps in ranking their stability in different formats and formulations but provides important mechanistic insights for improving the conformational kinetic stability of IgGs.

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By substrates modulated Hofmeister effect on catalytic activity of chymotrypsin

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Our study focuses on the effect of inorganic salts, Hofmeister salts, that specifically affect the enzyme activity of chymotrypsin toward the different substrates. The motivation of such study is to clarify the mechanism of Hofmeister effect and consequently, to improve the enzyme properties or the enzyme formulation for biotechnological applications.

Numerous works have been published about the Hofmeister effect of salts on properties of biomacromolecules but the mechanism of this effect remains unclear. There were developed several theories in the effort to explain the Hofmeister effect such as salt-in and salt-out interactions, water structure changes (low/high density water) and protein preferential hydration, and hydrophobic interactions. One of the first theories is the ion hydration characterizing by Jones-Dole viscosity *B*-coefficient. It has been found that salt solutions can be either more or less viscous than pure water depending on the nature of the salts. The ions with a positive value of *B* increased the viscosity of aqueous solutions and were supposed to "make the order" in the water structure. They were called "kosmotropes". Those with negative value of *B* decreased viscosity of aqueous solutions and were supposed to "break the order" in the water structure. They were called "chaotropes". Based on this classification of ions as kosmotropes and chaotropes, one possible explanation for Hofmeister´s experiments was that kosmotropic anions withdraw the water molecules of the hydration layer of proteins, thus forcing them to aggregate. Chaotropic anions were thought to act with exactly the opposite mechanism [1,2].

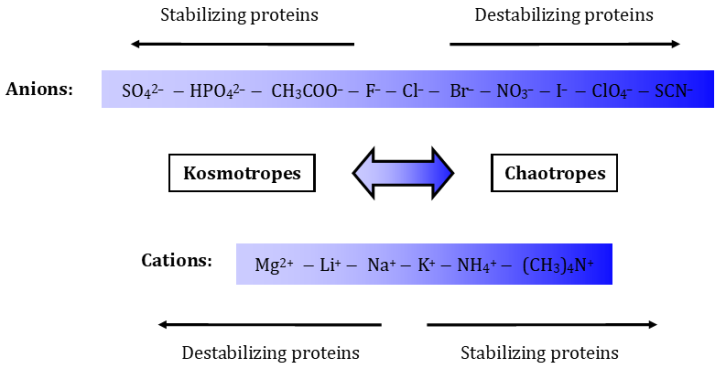

Fig. 1. Hofmeister series of ions.

One of the subjects of our study is chymotrypsin, which belongs to family of serine proteases that hydrolyzes the substrates after large hydrophobic amino acids residues (Phe, Tyr and Trp). This catalytic specificity is determined by the Ser residue at position 189 what is part of S1 binding pocket. Its tertiary structure is composed of two six stranded β-barrels and its active center contains catalytic triad composed of His57, Asp102 and Ser195, which is localized between those two domains (Fig.2) [3,4].

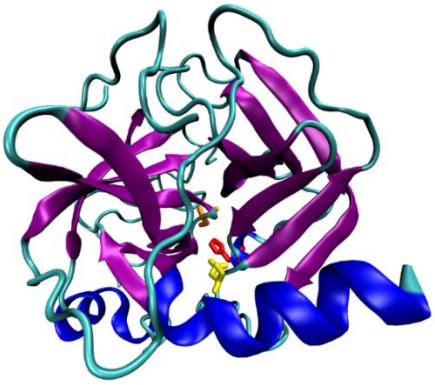

Fig. 2. The tertiary structure of chymotrypsin with active site: His57 (red), Asp102 (yellow) and Ser195 (orange). New cartoon representation made in program VMD 1.9.2.

We have studied the Hofmeister effect of anions on catalytic activity of chymotrypsin in dependence on three different substrates, BTEE (N-benzoyl-L-tyrosine ethyl ester), SPNA (N-succinyl-L-phenylalanine-p-nitroanilide) and GPNA (N-glycyl-L-phenylalanine-p-nitroanilide). Our results suggest that an extend of observed Hofmeister effect depends both on protein properties and on properties of substrates. We observed more significant Hofmeister effect on chymotrypsin catalytic activity in the presence of SPNA and GPNA compared to BTEE. The tendency of studied anions to affect the catalytic activity of chymotrypsin toward all three substrates was in accordance with the Hofmeister series.

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Characterization of conformational properties of AsLOV2 domain

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Plants, algae, bacteria and fungi contain Light-Oxygen-Voltage (LOV) domains that function as blue light sensors and control cellular responses to light. Blue light activation occurs when the non-covalently bound FMN chromophore absorbs a photon and forms covalent bond between its C4a atom and conserved cysteine residue within the protein (Fig. 1). After covalent bond formation protein undergoes a conformational change that results in the unfolding of the terminal Jα helix. This conformational change, is completely reversible in the dark [1,2]. Thanks to its light induced conformational change LOV domains have potential widepread use in optogenetics, i.e. as molecular switch in cell signaling and can also be used as a fluorescent protein when their advantage over GFP is smaller size and utility under anaerobic conditions.

Fig. 1. Schematic representation of LOV domain photochemistry. Blue light illumination leads to covalent bond formation between FMN and conserved cysteine residue. The photoreaction process is fully reversible in darkness [3].

For the purpose of this study we used two forms of LOV2 from *Avena sativa* (AsLOV2) domain: native form, so-called lit form, and the variant, so-called dark state form. In the dark form, photoreactive cysteine is replaced by alanine and this form thus does not undergo a photoreaction upon blue light illumination. Here, we present our findings regarding pH and thermal stability of AsLOV2 domain obtained by CD, fluorescence spectroscopy and differential scanning calorimetry. In addition, we investigated the effect of protein concentration and temperature on dark reversion kinetics of native AsLOV2.

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The use of DARPins in the purification of MBP-fused proteins

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DARPins (designed ankyrin repeat proteins) as a novel class of binding proteins with non-immunoglobulin scaffolds are in the center of interest of protein engineering research [1]. DARPins can be rapidly engineered to detect diverse target proteins with high specificity and affinity [2]. From this point of view, they offer an attractive alternative to antibodies. In this work, we study DARPin, which was selected with high specificity and affinity against maltose binding protein (MBP), with an intention to explore its application potential. MBP is a globular protein that is commonly used as a tag for MBP-conjugates purification with purpose to increase their solubility and expression yield [3]. The main goal of this study is to develop the affinity chromatography for MBP-conjugates purification based on interaction between the DARPin and MBP. In the effort to find optimal working conditions, we studied the influence of solvent pH on the stability of these proteins as well as on affinity of interaction between them. The results, we have obtained, suggest that our model of affinity chromatography has strong potential in purification of proteins containing MBP tag with comparable or higher expression yield of purified protein than commercially available affinity chromatographies.

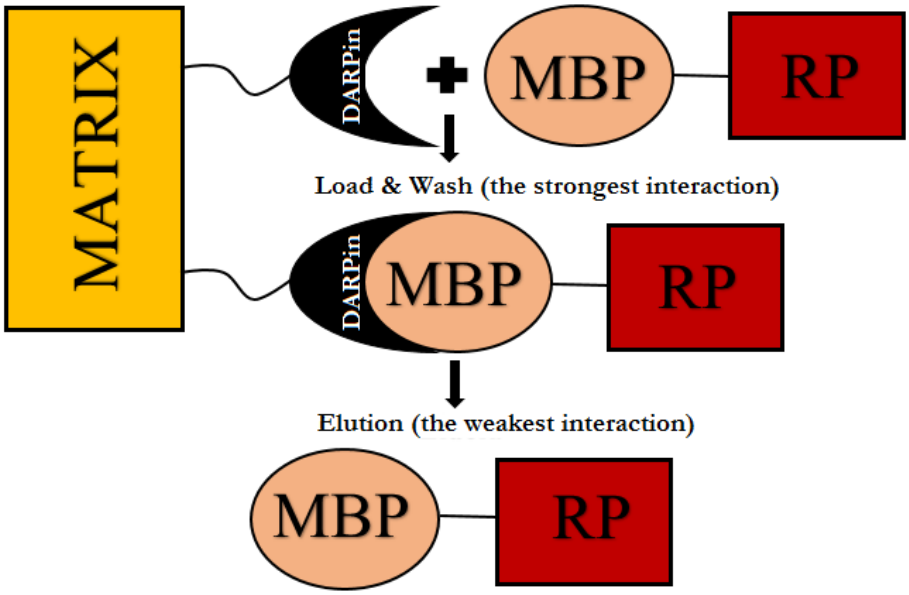

Fig. 1. A scheme of our designed affinity chromatography.

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Amino acids functionalized magnetic nanoparticles as lysozyme amyloid inhibitors

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Aggregation of protein into amyloid fibrils is a very important process in biology associated with a number of devastating diseases (Alzheimer, Parkinson, diabetes type II and other) [1]. Inhibition of amyloid fibrillization and clearance of amyloid fibrils are essential for the prevention and treatment of amyloid-related diseases. Recently, differently modified nanoparticles become one of the useful tools to target a number of medical complications [4]. There-fore, effect of nanoparticles on amyloid aggregation of proteins is intensively investigated [2, 3].

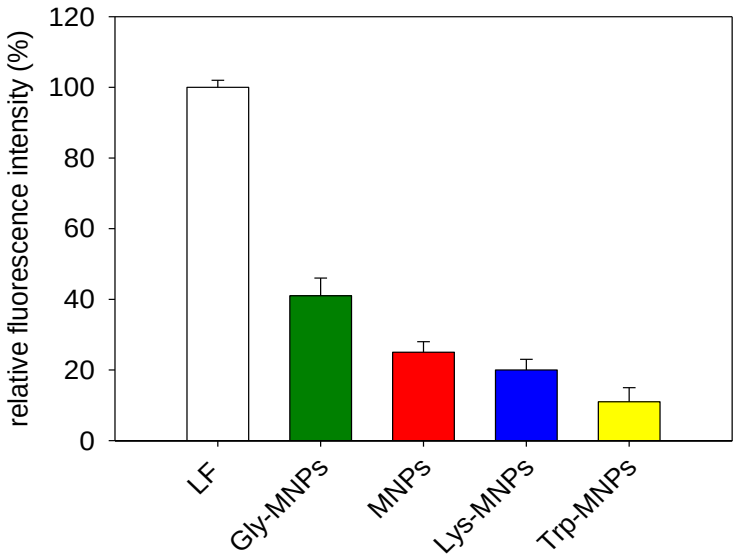

Fig. 1. ThT fluorescence measurement of lysozyme fibrils LF (white) and lysozyme fibrils in the presence of Gly MNPs (green), MNPs (red), Lys-MNPs (blue) and Trp-MNPs (yellow). Ratio of LF : all MNPs = 1 : 2.5.

In the present work we have investigated *in vitro* interference of lysozyme amyloid aggregation with bare Fe_3O_4-based magnetic nanoparticles (MNPs) as well as MNPs functionalized by three different amino acids, namely with polar lysine (Lys-MNPs), non-polar aliphatic glycine (Gly-MNPs) and aromatic tryptophan (Trp-MNPs) *o*. Using Thioflavin T fluorescence assay it was found that all studied nanoparticles are able to inhibit formation of lysozyme amyloid self-assembly as fluorescence intensities are lower compare to intensity determined for lysozyme fibrils (Figure 1). The observed inhibitory efficiency was in the order: Gly-MNPs (59%) < MNPs (75%) < Lys-MNPs 80% < Trp-MNps (89%).

To better understand the mechanism of nanoparticle inhibitory activities the kinetic of lysozyme fibrillization alone and in presence of all studied nanoparticles was investigated. The fibrillization kinetic of lysozyme alone displayed a sigmoidal profile typical for the formation of amyloid fibrils. Presence of studied MNPs leads to shortening of the lag phases and significant decrease of steady-state fluorescence intensities in the above mentioned amino acid order. The inhibitory effect of all studied nanoparticles was confirmed by atomic force microscopy.

Moreover, the effect of all our studied nanoparticles on the viability of human SH-SY5Y neuroblastoma cells was investigated. We found that studied magnetic nanoparticles are not toxic to the cells.

Based on the results, the presence of aromatic side chain rings on nanoparticle surface is an important factor determining the inhibitory effect of MNPs.

Acknowledgement
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Phosphorescence kinetics of singlet oxygen produced in nano-particles – modelling and experiments

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Oxidative stress induced by singlet oxygen inside living cells plays a key role in photodynamic therapy (PDT) of cancer [1,2]. Singlet oxygen, which is usually produced by energy-transfer between excited photosensitizer (pts) and ground state molecular oxygen, can be detected by measuring its phosphorescence at 1270 nm. To promote targeted pts delivery in the host body, various types of nano-carrier systems are studied extensively. The singlet oxygen phosphorescence kinetics produced by short laser-pulse photosensitization of pts inside nano-particles is influenced by diffusion of singlet oxygen in the heterogeneous environment [3]. In order to describe this effect, two theoretical models were developed: a more complex numerical one and a simple analytical one. These models predict the time-course of singlet oxygen concentration inside and outside of the spherical nano-particles following short-pulse excitation of the pts. A linear combination of the two concentrations (inside and outside the particle) multiplied with corresponding emission rate constants can be used to reproduce experimentally measured phosphorescence intensity of singlet oxygen in pts-loaded nano-carrier systems. The results of the models were applied to explain the phosphorescence kinetics of singlet oxygen produced by photosensitized hypericin inside of LDL particles.

Acknowledgement
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Spectroscopy and time-resolved microscopy study of endogenous fluorescence in marine algae *Dunalliela*

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Algae are aquatic organisms playing a key role in photosynthesis. In addition to their ecological roles as oxygen producers and as the food base for almost all aquatic life, algae are economically important as a source of crude oil, as well as sources of food and a number of pharmaceutical and industrial products. Deep understanding of algal cell response is essential to predict how ecosystems may be affected by the climate change and other human activities.

Our aim was to examine endogenous fluorescence (autofluorescence) by spectroscopy and microscopy methods and monitor cell behavioural changes under influence of stressors (salinity, heavy metals) for ecotoxic risk assessment in aquatic environment and bioremediation. We have chosen a marine alga *Dunalliela* [1], unicellular green algae, used extensively as model organisms for various research areas, as well as for mass culture, for these studies. We have previously demonstrated application of fluorescence spectroscopy and microscopy tools to record endogenous fluorescence in *Chlorella* algae [2], as well as its potential to be employed as a biosensing tool [3].

Non-invasive monitoring of processes underlying metabolic changes in living cells in their real environmental conditions is a prerequisite for understanding their pathophysiological changes. Among the brightest endogenous fluorophores inside biological tissue are chlorophylls in plants and NADP(H) and flavins in mitochondria. These molecules are the main electron donors and acceptors in the biochemical processes such as photosynthesis, or oxidative phosphorylation. Changes in autofluorescence thus reflect metabolic modifications in the living organism. We tested a possibility to record spectrally- and time-resolved autofluorescence in *Dunalliela* algae. Our first experiments tested the feasibility to evaluate the properties of endogenous fluorescence in these algae.

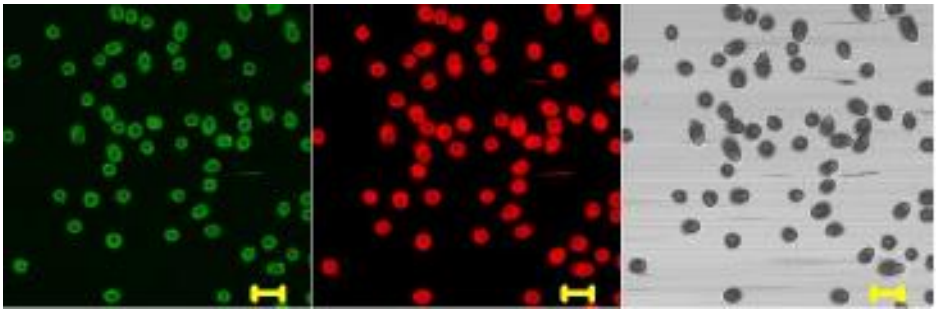

Fig. 1. Laser scanning confocal microscopy imaging of *Dunalliela* algae, exc. 458nm, green spectral region 480-520nm (left), red spectral region 620-680nm (middle) and transmission image (right), scale 20μm.

Emission spectra, recorded using spectrophotometer (Fluorolog 3-11 SPEX, USA) at excitation 375nm in the algae revealed main peak at 680nm, as expected for the peak of the chlorophyll, as well as an increased fluorescence emission between 420-540nm (data not shown). Based on these measurements, we have chosen two emission windows for recordings of spatial distribution of *Dunalliela* endogenous fluorescence using laser scanning confocal fluorescence microscopy (Axiovert 200 LSM 510 Meta, Carl Zeiss Germany, equipped with C-Apochromat 40x, 1.2 NA). First recording window was in the green spectral region, 480-

520nm, and the second one in a red spectral region, 620-680nm (Fig. 1), following excitation at 458nm. Intensity in the red spectral region was several times higher than that in the green one, as expected for the major contribution of chlorophylls. Gathered data confirmed recordable autofluorescence at both spectral channels, with spatial distribution mainly situated around the algae nuclei.

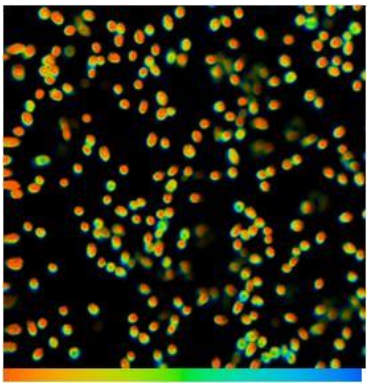

Fig. 2. Time-resolved fluorescence images of *Dunalliela* algae. Excitation 475nm, LP550nm, scale 500-700ps.

Time-resolved fluorescence imaging present a novel tool for evaluation of the sensitivity of the endogenous fluorophores to their environment and thus bring potentially very useful information for examination of changes in the algae responsiveness to stress conditions. Fluorescence lifetime images (FLIM) were recorded using time-correlated single photon counting (HPM 100-40 photomultiplier array (Becker&Hickl, Germany, employing SPC-830 TCSPC board, LP 500 nm) on fluorescence microscopy (Axiovert 200 LSM 510 Meta, Carl Zeiss Germany), following excitation at 475nm (BDL-475, Becker&Hickl, Germany). Algae fluorescence presented at least double exponential decays of 500 and 700ps, as represented at Fig. 2. For the first time, we thus demonstrate a possibility to record the FLIM images in the *Dunalliela* algae.

Gathered results on spectral and time-resolved characteristics of *Dunalliela* algae endogenous fluorescence demonstrate successful application of novel methodological approaches to evaluate the algae behaviour. In the future, physicochemical and structural characterizations of algal responsiveness will become of key importance to interpret and rationalize the behaviour of these cells under environmental stress conditions and to predict their fate in the aquatic systems.

Acknowledgement
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Poly(2-isopropenyl-2-oxazoline) as a tool for combined cancer immunotherapy

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Poly(2-alkyl-2-oxazolines) (POx) represent a very promising materials for different areas of biomedicine due to very low cytotoxicity, immunogenicity, and non-biofouling properties [1]. On the other hand, poly(2-isopropenyl-2-oxazoline) (PIPOx) prepared by radical polymerization is a functional polymer with reactive 2-oxazoline group in each structural unit (Fig. 1).

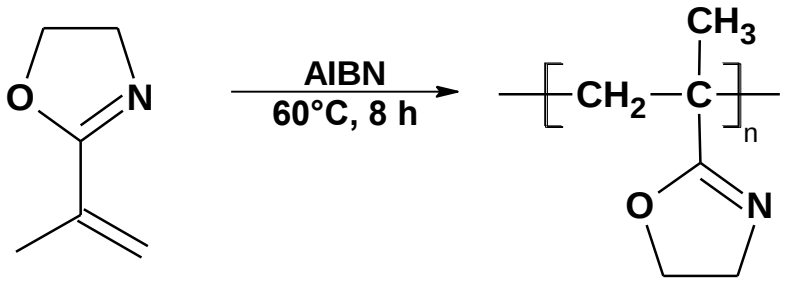

Fig. 1. Scheme of the free-radical polymerization of 2-isopropenyl-2-oxazoline initiated by AIBN.

Such type of synthetic polymers is currently attractive for different fields of biotechnology and medicine due to possible modification and conjugation with peptides, saccharides, or drugs. Recently, we showed that PIPOx is a biocompatible material and is suitable for biomedical applications (Fig. 2) [2].

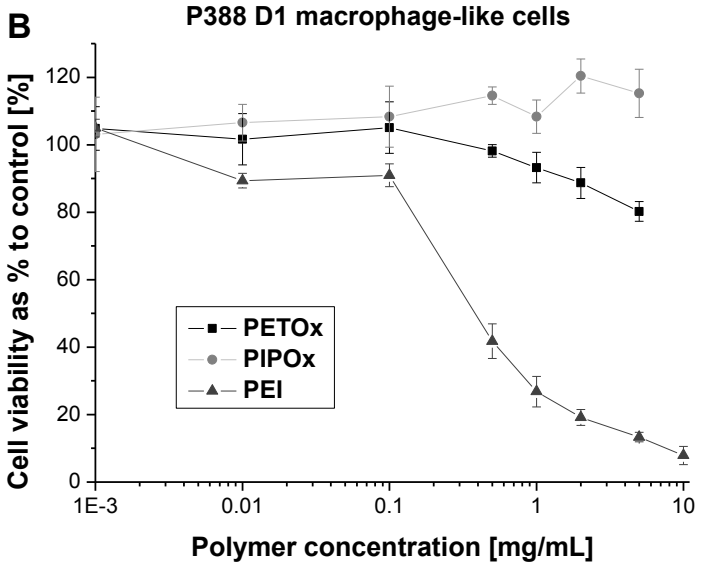

Fig. 2. In vitro toxicity of poly(2-ethyl-2-oxazoline) (PETOx) and poly(2-isopropenyl-2-oxazoline) (PIPOx) compared to polyethylene imine (PEI) as a positive control evaluated by a MTT test on mice macrophage cell line.

We demonstrated that PIPOx significantly stimulates proliferation of macrophages *in vitro* as well as ex vivo proliferation of non-adherent cells isolated from T-lymphocyte-enriched splenocytes following co-stimulation of PIPOX-induced adherent cells enriched in dendritic cells [1]. These results suggest that PIPOx may play a role in various immunomodulatory processes.

In vitro internalization of fluorescently labelled PIPOx to fibroblast and macrophage cell lines was studied by CLSM using organelles tracking dyes. Results indicate that PIPOx is mainly localized in lysosomes and other vesicles which are part of endosomal pathway.
PIPOx was also used for the conjugation with non-steroidal anti-inflammatory drugs (NSAIDs), which have been demonstrated as effective drugs in a cancer treatment. Conjugates with aspirin and ibuprofen exhibited enhanced toxicity toward cancer cell lines compared to non-conjugated drugs.

Acknowledgement
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Poly(2-oxazoline) gradient copolymer nanoparticles for curcumin loading and delivery to cancer cells

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Curcumin (CM), a naturally-occuring polyphenol, is renowned for its anti-cancer activity, however, its use as a viable drug is impeded by its low stability in water [1]. To address this, we have investigated the potential of amphiphilic gradient copolymers based on poly(2-oxazolines) (POx). POx may be considered as the next generation of the polymers in cancer therapy because of their tunable properties and biocompatibility [2-4]. Here we report on the synthesis of a POx based gradient copolymer and its self-assembly to prepare polymeric micelles for the targeted delivery of CM. Initial solvents of different polarity and hydrogen bonding ability used for dissolving either POx copolymer or POx/CM system plays an important role on the size, morphology and drug loading capacity of these nanoparticles. The chemical stability and solubility of CM is highly enhanced due to encapsulation inside micelles. CM within these spherical nanoparticles has become stable for one month after nanoparticles preparation. Moreover, we have investigated the cell cytotoxicity and uptake of these polymeric nanoparticles by two different cancerous cell lines (U87 MG and HeLa) to reveal a potential biomedical application of these nanoparticles.

Acknowledgement
This work was supported by the research grant from the Slovak Grant Agency VEGA No. 2/0124/18 and by the Slovak Research and Development Agency APVV-15-0485.

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Conformational transition of respiratory cytochrome c oxidase

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Membrane bound respiratory oxidases catalyze the four-electron reduction of molecular oxygen to H_2O. This redox reaction is associated with the generation of the transmembrane proton gradient. In spite of significant progress the identity of the structural elements involved in the proton translocation have not been fully elucidated.

The reduction of O_2 by bacterial and mitochondrial cytochrome *c* oxidases (CcO) is facilitated by four redox active centers, Cu_A, cytochrome *a*, cytochrome a_3 and Cu_B [1]. Clearly, at least one of these four redox centers should provide the thermodynamic driving force for the transmembrane proton transfer. Several alternative models of the redox-linked proton pumping have been suggested. A common feature of these models is a redox dependent conformational cycling between the proton-input state and proton-output state. These two conformations should be present in both oxidized and reduced forms of the participating metal center.

Here we explore in more detail the availability of two conformational states for individual cytochrome *a* and a_3 of bovine heart CcO in both oxidized and reduced states. The optical spectra of heme cofactors were utilized as indicators of the structural state of the both cytochromes. To increase the sensitivity of detection of possible conformational changes the second-derivative absorption spectroscopy was applied [2].

Our data revealed the redox-dependent transition in the structure of cytochrome *a*. It appears that the reduction is associated with the increase of the polarity in the immediate surroundings of heme of cytochrome *a*. We suggest that the major determinant of this change of polarity is water access to the heme via the open channel in the ferrous cytochrome *a*.

Acknowledgement
This work was supported by Slovak grant agency (VEGA -1/0464/18) and the Slovak research and development agency (APVV-15-0485).

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The effect of central helix mutations on the stability of the N-terminal region of the cardiac ryanodine receptor

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Mutations in the cardiac ryanodine receptor calcium release channel (RyR2) are the cause of several inherited cardiac arrhythmias, but the mechanism of their effect is not clear. We have examined a mutation cluster in the central helix of the N-terminal region (NTR) of RyR2, which contains five mutation sites, in which 7 different arrhythmogenic mutations have been found. Using the X-ray structure of the human RyR2 NTR [1] as a template, we have created structural models of the NTR in the closed and open conformation for the wild type (WT) human RyR2 and for the 7 mutations (Fig. 1). The models were compared to those of a hypothetical complex of NTR with the domain peptide DP_{cpvtN2} that activated RyR2 under diastolic conditions [2].

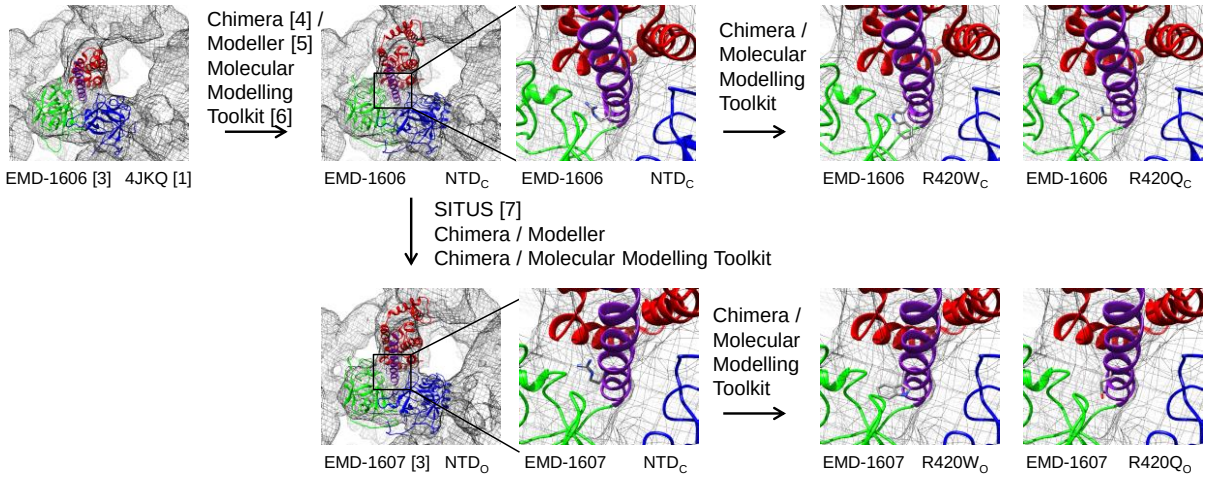

Fig.1. Construction of models of the closed and open NTR from human RyR2. Software used for individual steps is indicated.

Mutated NTRs contained less H-bonds than WT NTR and therefore were energetically less favourable. In contrast, the NTR-DP_{cpvtN2} complex was energetically more favourable than WT NTR [2] due to creation of interaction networks between the NTR and DP_{cpvtN2}. The energy of the closed-to-open transition, determined by energy minimization *in vacuo*, was in 6 of the 7 mutations lower than in the WT NTR (Fig. 2), as has been previously observed for the NTR-DP_{cpvtN2} complex [2]. In the mutated NTRs, the larger relative stability of the open conformation was due to a smaller mutation-induced decrease of the number of H-bonds between amino acid side-chains in the open (by 3 ± 0.7) than in the closed conformation (by 21± 2.7). In the complex, it was due to disruption of the central interaction network within NTR in the closed but not in the open conformation [2]. The activating effect of DP_{cpvtN2} binding and of mutations

in the central helix thus might be explained by their similar effect on the transition energy between the closed and open conformation of RyR2 NTR.

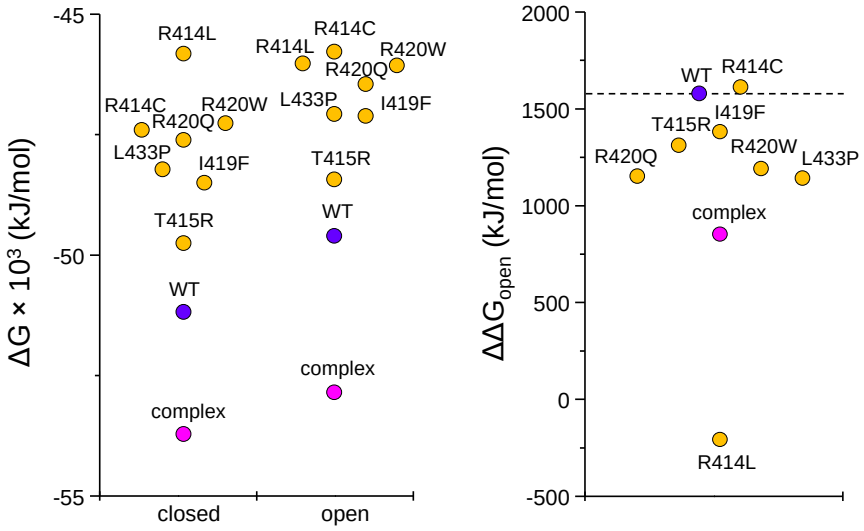

Fig. 2. Left: Comparison of the energies of the closed and open conformation of mutant NTRs (orange) with those of the wild type (blue) and of the complex of NTR with the domain peptide DP_{cpvtN2} (magenta). Right: The energies of closed-open transition. The data for the complex are taken from [2].

In the presentation we will compare the results of equilibrium calculations *in vacuo* to estimation of the thermodynamics of the closed and open NTR conformations *in aqua*, using free energy perturbation methods in NAMD [8].

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On the thermal dependence of the electrical capacitance of cell membrane

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Irradiation by brief infrared laser pulses causes excitation of the neural tissue in vivo [1, 2] and in vitro [3], as well as of cardiac tissue in vivo [4, 5]. The effect was attributed to thermal effects due the water absorption of the short wave infrared radiation [6]. The specific molecular target was not identified, although ion channels were considered as likely candidates. Shapiro et al. [7] showed that the excitation comes from the transient increase of electrical capacitance of the cell membrane. The increase of membrane capacitance was confirmed in model lipid membranes and various cell types in vitro [7], including cardiac myocytes [8]. The experiments were supported by Gouy-Chapman-Stern theory of charged surfaces used to model the effect of temperature on the membrane surface electrical double layer (Fig 1) [7]. This allowed Shapiro et al. to conclude that the temperature increase causes increase of cell membrane capacitance, which leads to redistribution of ions causing displacement currents that locally depolarize the membrane [7]. The depolarization secondarily activates voltage dependent ion channels and gives rise to initiation and spreading of action potentials. However, there was a caveat, as pointed out by Plaksin et al. [9]. These authors revealed contradictions in the proposed theory claiming that, from the energetic considerations, the thermal energy caused increase of capacitance should be offset by higher electrical energy and absolute surface potentials. These effects should result in decrease, not increase of capacitance. This decrease is further compounded by the decrease in dielectric constant of water at increased temperature. Reanalyzing the model of Shapiro et al. they found that, indeed, according this model the capacitance should decrease, not increase (Fig 1d), if a mathematical convention error was not involved [9]. In the revised model, Plaksin et al. [10] incorporated also other physical factors that vary with temperature. Their model provided quantitative agreement with the experimentally observed increase in membrane capacitance at elevated temperatures (Fig 2a) and attributed the effect to reduced thickness of the membrane bilayer and to increased area per phospholipid molecule (Fig 2b). The explicit calculations directly confirmed universal experimental findings on the thermal effects of laser pulse irradiation on membrane capacitance and allowed the original estimation of the membrane surface charge difference that gives rise to action potential initiation.

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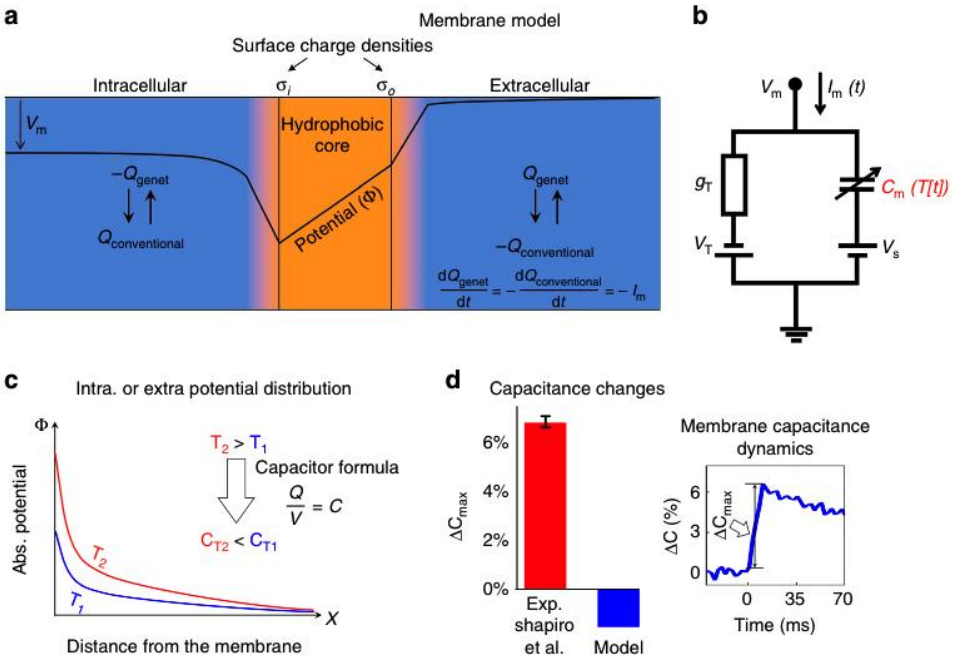

Fig. 1.* Predicted membrane electrical capacitance after temperature increase. **a** Theoretical Gouy–Chapman–Stern model with phospholipid (hydrophobic) core and extra-membranal boundary sub-regions. The model is shown under conventional charge notation (Boron and Boulpaep *Medical Physiology*, Elsevier Health Sci, 2016) and the non-standard notation of Genet et al., *Acta Biotheor*., **48**, 2000, 273, where the charge derivative has opposite direction to the current convention. **b** Membrane-equivalent electrical circuit reproduced from Shapiro et al. (2012); the membrane current is marked according to the conventional convention. **c** Illustration of potential distribution at the extra-membranal boundary regions. Temperature elevation leads to higher potential gradients, which according to the classical capacitor formula corresponds to a reduction in capacitance of the extra-membranal regions. **d** Discrepancy between capacitance measurement (reproduced from Shapiro et al. (2012) and sign-corrected model simulations (PE:PC bilayers1, laser parameters: 10 ms, 7.3 mJ)

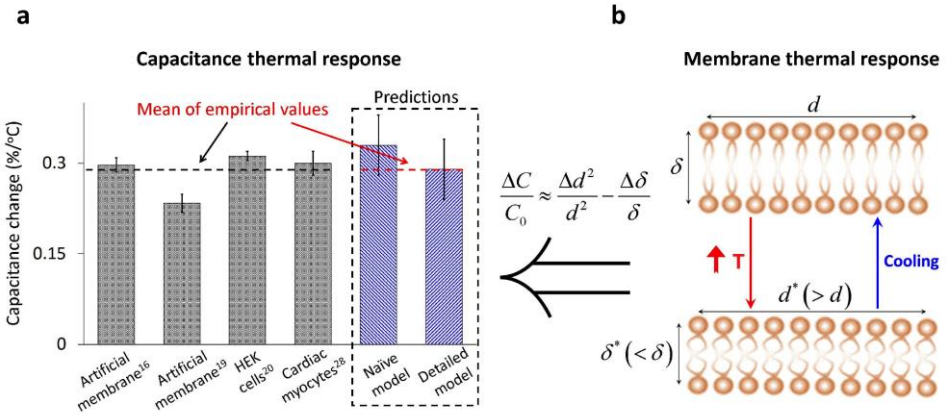

Fig. 2.* Universal membrane capacitance thermal response rate, explained by membrane dimensional changes. **a** Membrane capacitance change rates measured in different studies and preparations (gray bars) vs. predictions from the thermal dimensional response of POPC bilayers (Naive model), and from a detailed biophysical model accounting for spatial charge distribution. (b) Schematized membrane thinning and area expansion under temperature elevation. This observed process is thought to result from an increase in the fatty acid chains trans–gauche rotational isomerization, which shortens the tails effective length (δ) and increases the area per phospholipid molecule (d). Biophysically, these two phenomena contribute to a predictable increase in both the membrane hydrophobic core and total capacitance. Error bars for the direct capacitance measurements are ±s.e.m, and for the model predictions are ± chi-squares distribution-related uncertainty.

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Early diagnostics and therapy of gastrointestinal neoplasia (from diagnostics to therapy within 15 minutes)

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New approaches to the early detection and resection of gastrointestinal neoplasia will be presented. Neoplasia of gastrointestinal tract - polyps – are formed from neoplastic cells with high probability of turning into a colorectal carcinoma. The main result of my work is a contrasting solution able to penetrate the bioptic channel (small flexible hollow tube, several meter long, with attached endoscopic camera and surgical loop), commonly used for detection and, in case of positive finding – removal of localized tumor of gastrointestinal tract. Instead of few-seconds hunting in visually challenging environment where gastroenterologist tries to see and catch slightly elevated tissue into a bioptic loop for cutting, our approach with directed diffusion of multicomponent solution will cause significant elevation and coloring of polyp against neighboring tissue, so that the surgeon clearly sees through camera the polyp itself, the boundary layer separating the polyp from healthy tissue as well as the neighboring healthy tissue itself. The polyps not grown deeply through *muscularis propria* will form temporarily narrow stem, suitable for cut. The surgery can be done in more comfortable time as we are able to prepare the solution so that the stem and polyp remain ready for resection for 15 or more minutes. The solution facilitates resection of an early stages of neoplasia, but primary after an injection allows to determine also the stage of neoplasia, based on the stem width and stem color. This simple visual control allows to see the level of embedding of neoplasia into underlying structure and thus serves also as diagnostics and preventive tool.

The incidence of gastrointestinal polyps, because of western lifestyle, is surprisingly high. Therefore, we can say with confidence, that present population might directly benefit from our solution as the patients.

The solution received an award “Innovation of the year 2017” in Slovak republic, attributed Certificate of registration of the utility model of the Slovak republic [1]. The international patent application is pending [2].

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X-ray bioimaging using Bragg Magnifier Microscope

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We present an alternative method for 3D tomographic imaging of microscopic biological objects in hard X-ray regime, based on Bragg Magnifier (BM) principle [1]. BM microscope uses asymmetrically cut Germanium crystals to magnify X-ray beam with advantages such as shorter propagation distances and increased dose efficiency, while achieving decent spatial resolution. This work focuses on the 3D reconstruction method to interpret the X-ray tomographic holograms using single-distance phase retrieval algorithm developed specifically for Bragg Magnifier, which is followed by filtered back-projection. We use a modification of contrast transfer function approaches developed for propagation phase-contrast based imaging and in combination with iterative constraint-based phase retrieval algorithm we obtained faster and more robust reconstruction method [2]. Our algorithm was successfully applied to both synthetic and real-world experimentally measured holograms as demonstrated on 3D electron density reconstruction of model organism Tardigrade (Fig. 1). We reached isotropic spatial resolution 300 nm approaching theoretical resolution limit for the given experimental setup.

We also propose an algorithm for efficient and accurate free space 2D wave propagation through the set of inclined reflecting planes (for the case of X-rays, the planes represent crystals in the Bragg geometry) [3]. The direct application of this method is found in fast and precise forward and backward propagations through the Bragg Magnifier Microscope setup.

Acknowledgement
This work was supported by the research grant VVGS-PF-2015-470.

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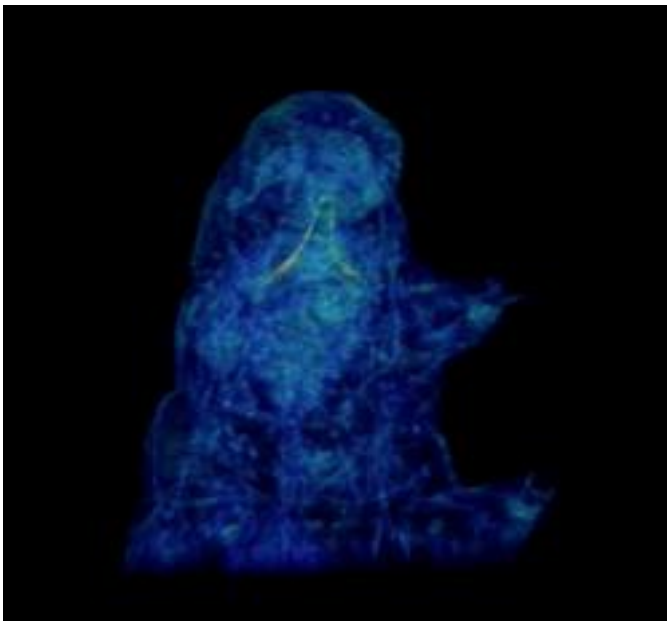

Fig. 1. 3D rendering of the image of Tardigrade obtained using Bragg Magnifier Microscope.

Developement of GPU accelerated ray-tracing based X-ray simulation enviroment

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Introduction: A framework for simulating deterministic mono- or polychromatic X-ray beam transfer in computed tomography (CT) through arbitrary geometrical objects has been developed.

Materials and Methods: For performance reasons a GPU-based acceleration has been applied that scales automatically from personal to high-end workstations. Detailed CT-scanner geometries and acquisition trajectories are supported and can be adapted freely. Multiple object geometries (e. g. spheres, cylinders, cones, boxes, etc.) with support of object motion can be defined in 3D space with arbitrary transformations either as pure material, user-defined mixtures or compounds. Specific attenuation phenomena can be selected and studied individually (e. g. the sole effects of Compton-scatter). Resulting projection data are stored as sinogram datasets to enable further analysis and image reconstruction. Result: GPU-accelerated simulation of various CT-scanner geometries, object scenes and acquisitions is feasible and could help to reduce simulation time and study more complex hardware configurations (e. g. counting detector system, inverse geometry) and sophisticated acquisitions (e. g. 4D perfusion, radiation therapy) in the future.

POSTERS

A novel approach to treating Alzheimer's disease? Photoswitchable molecules

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The presence of $A\beta_{42}$ amyloid fibrils is one of the central pathological hallmarks in Alzheimer's disease (AD). Therefore inhibiting aggregation and disruption of fibrils of $A\beta_{42}$ peptide may be an effective strategy for combating this so far incurable disease [1]. One of the recent therapeutical strategies is focused on the inhibition and clearance of amyloid fibrils using small molecules. Hydrophobic small compounds containing aromatic groups have high potential for Aβ binding and subsequent reduction of the amount of amyloid aggregates [2].

In our research we focused on azobenzene derivatives as azobenzene is the most common studied molecule that undergo reversible isomerization between the *cis* (non planar) and *trans* (planar) conformations, which can be switched with ultraviolet or blue light. Our objective was to explore the anti-amyloid properties of azobenzene molecule and its derivatives and how isomerization will influence their anti-amyloid properties. The effect of compounds on $A\beta_{42}$ fibrils was studied using Thioflavin T assay in broad concentration range and DC_{50} values were determined.

Data from ThT assay have shown that azobenzene derivatives are able to disrupt $A\beta_{42}$ fibrils with DC_{50} in stoichiometric concentration when incubated in dark conditions. Without light the azobenzene is in its planar form and can bind to fibrils most likely by intercalation between β strands of fibrils. Upon irradiation, the destroying activity of azobenzene derivatives switched to *cis* conformation was slightly higher. This suggests that after intercalation we can enhance the disruption of fibrils by switching of the structure of these molecules. Our results were supported by atomic force microscopy and docking methods.

These results open up a new world of possibilities for designing better treatment agents for AD and hopefully also other amyloid-related diseases.

Acknowledgement
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Study of the influence of negatively charged mixed micelles on properties of cytochrome c

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Cytochrome *c* (cyt *c*) is known not only for its electron transfer activity in electron transport chain in mitochondria, but also for its function as initiator of apoptosis. It is generally acknowledged that cyt *c* function in apoptosis is accompanied by its increased peroxidase-like activity, which is triggered by its interaction with negatively charged inner mitochondrial membrane [1]. In the presented research, we attempted to determine amount of negative charge, which is necessary to induce the peroxidase activity as well as conformational changes in cyt *c* induced by interaction with the negatively charged surface.

The interaction of cyt *c* with negatively charges membrane surface is of electrostatic and hydrophobic natures and triggers methionine decoordination and formation of non-native low spin, and high spin forms of cyt *c* [2]. For understanding of structural modifications of cyt *c* induced by interaction with membrane surface, we used, as a model system, mixed micelles containing spectroscopically silent dodecyl maltoside (DDM) and sodium dodecyl sulphate (SDS). This approach allowed us to assess a role of electrostatic interaction in conformational changes of cyt *c*. The effect of mixed micelles (SDS:DDM) on conformation, pK_a, T_m values, and peroxidase-like activity of cyt *c* was analyzed by absorption spectrometry (Soret, 620 nm and 695 nm), fluorimetry (Trp at 350 nm) and spectropolarimetry (Soret, aromatic and peptide region, 222 nm) in neutral pH. Modification of the charge of micelles was achieved by modification of ratio SDS:DDM (1:1, 5:1, 10:1, 15:1, 30:1 and 60:1) at two different concentrations of micelles: 0,5 and 2,5mM.

Our results indicate that the peroxidase-like activity of cyt *c* significantly increases in the presence of the mixed micelles with negative charge of about 10-15. Interestingly, at these conditions, the conformation of cyt *c* was not significantly affected what suggests that the increased peroxidase-like activity of cyt *c* is associated with an increased dynamic of its heme region.

Acknowledgement
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Changes in calcium dynamics and contractility of left ventricular myocytes isolated from Wolframin1-deficient rats

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Background & Aim

Wolfram syndrome (WS, OMIM 222300) is a rare hereditary disorder characterized by diabetes insipidus, diabetes mellitus, optical and brain stem atrophy, and deafness. Mutations in Wolframin1 (Wfs1) gene, which encodes protein harbouring endoplasmic reticulum (ER) membrane are responsible for WS. Wolframin1 has been shown to positively modulate Ca^{2+} level in the ER by increasing the rate of Ca^{2+} uptake [1]. Moreover, it has been demonstrated that the WFS1 co-immunoprecipitates with sarcoplasmic-endoplasmic reticulum calcium ATPase (SERCA) and that it negatively regulates SERCA expression [2]. All this evidence demonstrates impact of WFS1 to calcium metabolism.

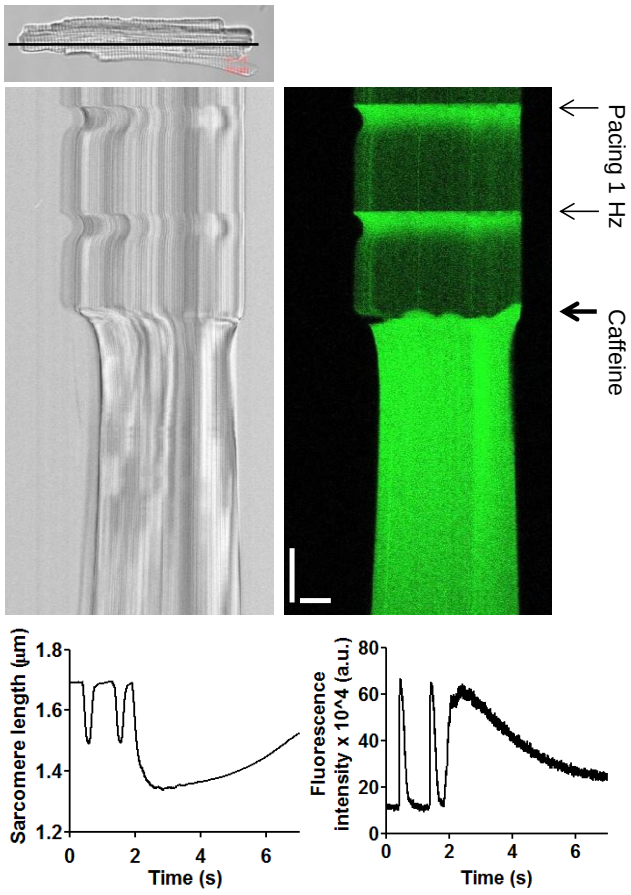

Fig. 1. Simultaneous acquisition of shortening (left side) and calcium transients (right side) of Fluo-3 labelled cardiac myocyte. From the brightfield image (top) a line was selected and time lapse was recorded simultaneously in transmission and fluorescence channel. Bottom: temporal development of sarcomere length and Fluo-3 fluorescence after pacing at 1.0 Hz. Bars: 0.5 s; 10 µm.

WFS1 is highly expressed in the pancreatic β-cells, neurons and cardiac myocytes. Although clinical evidence depicting cardiac symptoms in WS increases, the role of Wfs1 for cardiac myocyte function was not determined. Since calcium ions are the trigger for myocyte contraction, in this work we evaluated calcium transients and contractility of left ventricular myocytes isolated from the exon5-WFS1 deficient rats.

Methods

Experimental model. To study impact of WFS1 to human disease, our collaborative group at the University of Tartu has developed the loss of function (exon5 deficiency) rat model. To determine the impact of the Wfs1 malfunction on diabetes independent remodelling of calcium dynamics in cardiac myocytes, the animals at the age of four months were used.

Isolation of cardiac myocytes was performed as previously described [3].

Confocal imaging. The TCS SP-8 STED confocal microscope (Leica Microsystems) was used for the study. Myocytes were loaded with the calcium indicator Fluo3-AM for 15 min at room temperature. Contractility was acquired in transmission simultaneously with Fluo3 fluorescence (excitation at 500 nm, emission at 510–570 nm). Fluo3-labelled myocytes were perfused at a rate of 1 ml.min^{-1} and paced to contract at a frequency of 1.0 Hz using field stimulation. A rapid line scan confocal imaging protocol (2.5 ms per line) was used to record contractions and calcium transients quasi-simultaneously (Fig. 1). Calcium content of the sarcoplasmic reticulum was probed by rapid perfusion with 20 mM caffeine.

Data processing/Statistical analysis. Images were processed using the SarConfoCal plugin [4] of the Fiji image processing software [5] and further analysed with Clampfit (Molecular Devices). Contractility was defined as fractional cell shortening, calcium transient as fractional increase of Fluo-3 fluorescence intensity. Statistical analysis was performed in Origin (OriginLab) using Student's t-test.

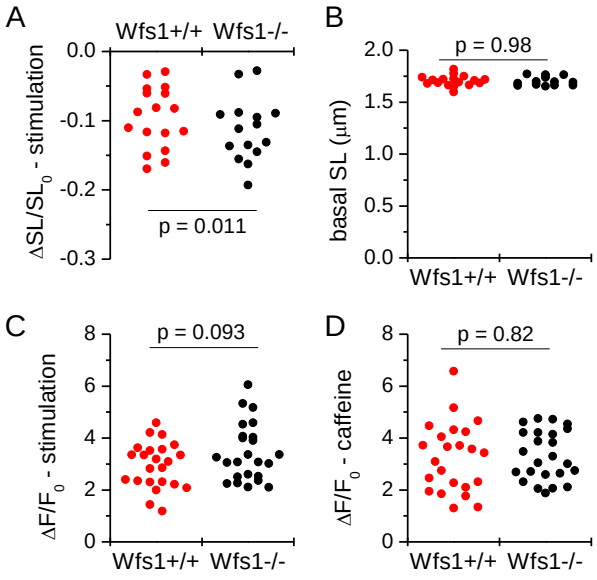

Fig. 2. Calcium transients and contraction in control and Wfs1-/- myocytes. A - fractional decrease in sarcomere length; B - basal sarcomere length; C - calcium transient in response to field stimulation; D - calcium transient in response to caffeine application.

Results

Wfs1-/- animals showed significantly increased cell shortening in response to field stimulation (Fig. 2A, 9.6 ± 1.1 and 3 ± 1.2 % for Wfs1+/+ and Wfs1-/-, respectively, $p < 0.02$). The amplitude of calcium transients had also an increasing tendency (Fig. 2C), but the increase did not reach statistical significance ($\Delta F/F_0 = 2.94 \pm 0.18$ and 3.44 ± 0.22 for Wfs1+/+ and Wfs1-/- respectively, $p > 0.05$). There was no significant change of basal sarcomere length (Fig. 2B) or of the amplitude of the caffeine-induced calcium transient (Fig. 2D).

Conclusions

We conclude that the absence of functional WFS1 in left ventricular myocytes results in a decrease in myocardial contractility that might be a result of perturbed calcium homeostasis. Our data implicate for the first time that WFS1 function and amplitude of cell shortening and calcium transients are negatively correlated in left ventricular myocytes. This is in accordance with the postulated negative correlation between expression of WFS1 and that of SERCA [2].

Acknowledgement

The work was supported by the SASPRO grant No. 0063/01/02, financed by the Slovak Academy of Sciences and by a "Co-financing of regional, national and international programs (COFUND)", which is part of the Marie Curie Action of the EU 7th Framework Programme, under Grant Agreement No. 609427; VEGA 2/0169/16; APVV 15-0302 CAMYS; and by the project "Completing the infrastructure for modern research of lifestyle diseases " (ITMS 26230120006), funded by ERDF.

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The use of the quail chorioallantoic membrane as an experimental *in vivo* model for photodynamic applications

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The chorioallantoic membrane (CAM) is a simple, highly vascularized extraembryonic membrane, which performs important functions during development of an avian embryo. CAM model offers multiple advantages over other *in vivo* models used to study normal and tumor angiogenesis. The chorionic epithelium is very similar to human epithelium and has a collagen IV rich basement membrane. Chicken CAM is used most often, however another feasible model is the quail chorioallantoic membrane. We have successfully used it as an *in vivo* model for the study of various biological processes. A quail embryo is cultivated using *ex ovo* technique. Tested substances can be applied systemically, or topically on the whole area of CAM, or into the silicone ring. We have tried angiogenic effect of several compounds, e.g. endogenous peptide leptin administered *in ovo* or on *ex ovo*. Changes in blood vessels were quantified by the fractal analysis. Photodynamic therapy (PDT) is a promising and innovative treatment for small localized tumors. PDT is based on the concept that tumor destruction occurs when a photodynamically active molecule, photosensitizer, accumulates within the tumour and is consequently activated by light. We have focused on photodynamically active drug Hypericin (HYP). HYP was applied topically on quail CAM to study cancer diagnosis and treatment. We applied Hyp in combination with low density lipoproteins(LDL),which appears to be promising delivery system for hydrophobic antitumour drugs.

Human oesophageal squamous cell carcinoma (TE1) spheroids were implanted on CAM surface on embryonal day 7 and after 24 hours formulations of free Hyp and Hyp:LDL 100:1 and 200:1 were topically applied. Both Hyp and Hyp:LDL formulation very well visualized the tumour spheroid position in fluorescent light. Our results also demonstrate that Hyp:LDL complex produces a higher fluorescence intensity and better image contrast compared to Hyp alone. Gathered preliminary results indicate that, thanks to the transparency of its tissues, quail CAM model can be useful for research of angiogenesis, for development of novel biophotonic techniques and for investigation of the effects of photodynamically active drugs. In other series of experiments, we have simulated a microbial infection on CAM tissue. Yeast inoculation had no effect on embryo survival. Penetration of Hyp into yeast cells was slower compared to tumor cells. This may be explained by yeast colony metabolic and morphologic conditions, which attenuate drug penetrability and permeability. The addition of HYP and HYP:LDL improved the visualization of yeast infection.

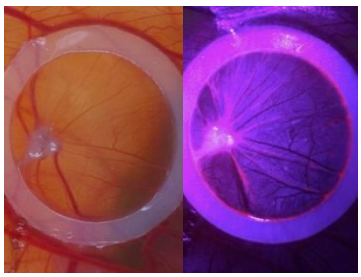

Fig. 1. TE1 tumor after 6h in blue excitation light after HYP:LDL administration

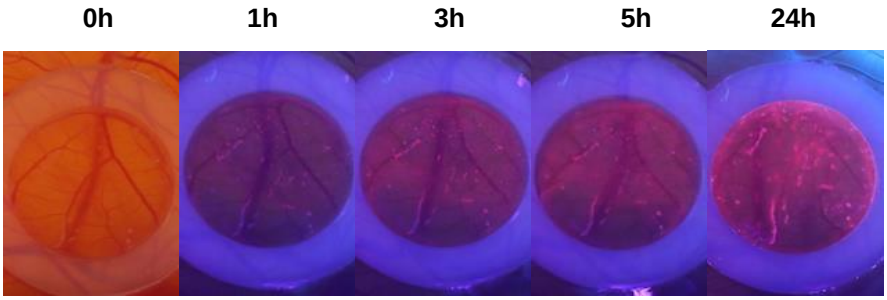

Fig. 2. S. cerevisiae on CAM in blue excitation light after application of HYP:LDLin time slots.

Conclusions

Quail CAM is accessible and fast *in vivo* prototype for research and understanding of the tumor angiogenesis, antivascular and antimicrobial therapy. Our experimental results confirm that Hyp and Hyp:LDL complex is potent fluorophore for photodynamic diagnosis of carcinoma and yeast infections.

Acknowledgement
This research was supported by VEGA grant 2/0096/18 and APVV-15-0485.

Telomeric G-quadruplexes and their derivatives: From human to *Tehrahymena* repeats

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G-quadruplexes are structures with high level of organization formed from guanine rich nucleic acid sequences. Sequences able to form G-quadruplex structures were found in eukaryotic telomeres and also in another important regions of human genome, e.g. gene promoters [1].

The human telomeric and protozoal telomeric sequences differ only in one purine base in their repeats; TTAGGG in telomeric sequences; and TTGGGG in protozoal sequences. In this study, the relationship between G-quadruplexes formed from these repeats and their derivatives is analyzed and compared. The human telomeric DNA sequence (HTR) $G_3(T_2AG_3)_3$ and related sequences in which each adenine base has been systematically replaced by a guanine were investigated; the result is *Tetrahymena* repeats (THR). The substitution does not affect the formation of G-quadruplexes but may cause differences in topology. The results also show that the stability of the substituted derivatives increased in sequences with greater number of substitutions. In addition, most of the sequences containing imperfections in repeats which were analysed in this study also occur in human and *Tetrahymena* genomes [2].

In this study, we clearly demonstrate that increasing the number of guanines in the loop regions of HTR sequences supports the formation of G-quadruplex structures. Any substitution of A-for-G increases the melting temperature, while the introduction of several substitutions was found to facilitate the coexistence of several conformers in the presence of potassium. The systematic introduction of these substitutions finally leads to the formation of sequences which occur in the *Tetrahymena* telomere. Besides, the structure of THR shows some structural features which are different from those of HTR and HTR derivatives in the presence of potassium. Generally, the presence of G-quadruplex structures in any organism is a source of limitations during the life cycle. Therefore, a fuller understanding of the influence of base substitution on the structural variability of G-quadruplexes would be of considerable scientific value [2].

Acknowledgement
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Raman and SERS characterization of iron gall inks: preliminary results

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Iron gall inks (IGIs) were historically the most important and common writing material until the beginning of the 20th century [1]. They have also been used to prepare many for humanity important historical documents and artefacts [2]. They consist of a mixture of gallnut extracts and vitriols (sulfates of certain metals) agglutinated by gum Arabic (Figure). However, the specific recipe varied depending on the region, culture and/or historical moment. The corrosive nature of IGIs speeds up degradation of the paper and alters the chemical properties of the ink producing changes in color and brightness. Although iron is believed to be one of the main factors triggering cellulose degradation mechanisms [3], the precise mechanism of ink decomposition is still unclear and a specific combination of the used ink and substrate together with the storage conditions have also to be considered. Thus, analysis of IGIs can reveal useful information not only about the origin of the inks and authenticity of historical documents, but also about the assumed degradation processes. In consequence, it also helps to acquire more precise information about the optimal artefacts' preservation and conservation.

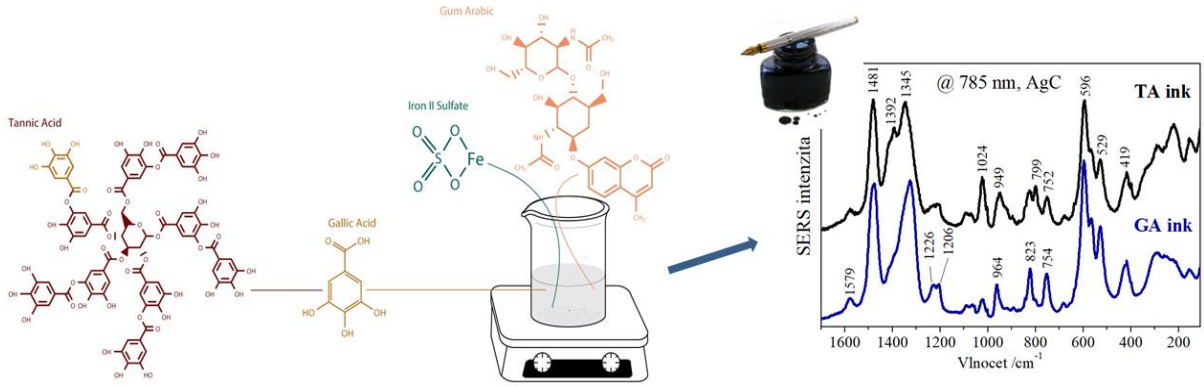

Fig. 1. Representative image of the Iron-Gall Ink preparation depicted with the chemical structures of the ink's principal components and their corresponding SERS spectra

The standard analytical methods are based on chromatographic techniques [4, 5], which are highly sensitive and selective, but imply sampling. They are destructiveness. Raman spectroscopy (RS) has evolved as an efficient non-destructive, and even *in situ*, technique for identification of inorganic pigments and other materials in artworks [6, 7]. However, the application of RS is limited when applied in the study of natural organic pigments and dyes caused by the enormous fluorescence displayed by these compounds and the low sensitivity of the technique. Surface enhanced Raman scattering (SERS) can overcome these problems, because of the fluorescence quenching induced by nanostructured metal surfaces in addition to the large enhancement produced by the surface localized plasmon resonance at appropriated excitation lines [8]. It has also been shown that SERS as an *in situ* technique with high sensitivity achieves results comparable to those obtained by chromatographic methods [9]. Although SERS seems to be a good technique for analyzing different artefacts, there are only a few studies of paper-based artefact analysis [1, 10-15]. Moreover, Raman spectroscopy for

systematic study of IGIs has been used only exceptionally [16, 17] and as far as we know there is no work concerning SERS spectra of IGIs and their interpretation. However, Kurouski *et al.* used TERS for *in situ* identification of IGIs on paper [18].

The present work is focused on the characterization of different IGIs by means of Raman and SERS techniques. We have prepared IGIs on the base of traditional recipes using either gallic or tannic acid [19, 20] and characterized them spectroscopically. Preliminary results allowed us to determine the optimal conditions for the IGIs' SERS detection (AgC colloid, λexc = 785 nm, pH ~ 4.5). Further, we have recorded SERS spectra of IGIs at low concentration (~ 10^{-7} M). Our aim is/was to prepare, identify and characterize the IGIs with compositions as close as possible to the real ones (used in ancient manuscripts and documents) and thus, to be able to detect and evaluate deterioration observed in different artefacts and to understand the undergone degradation processes.

Acknowledgement
This work is supported by the Scientific Grant Agencies of the Ministry of the Education of Slovak Republic (VEGA 1/0929/16 and APVV-15-0485).

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Raman and surface–enhanced Raman scattering analysis of lichen *Xanthoria parietina*

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Raman spectroscopy is an important method for investigation of various plant tissues because provides molecular level information on composition and structure of cellular components [1-3]. One of the main problems associated with the use of conventional Raman spectroscopy for the study of biological materials is the very strong autofluorescence that is produced when phenolic compounds are excited by visible light. Near-IR excitation leads to a dramatic decrease of plant tissue auto-fluorescence, on the other hand, however, generates a weak Raman signal. Surface enhanced Raman scattering (SERS) can overcome these problems because of the fluorescence quenching, induced by nanostructured metal surfaces, together with the large enhancement of the signals produced by the surface localized plasmon resonance [4].

Lichens are symbiotic organisms composed of mycobiont (fungus) and photobiont cells (algae or/and cyanobacteria). The photobiont possess the chlorophyll and the photosynthetic apparatus necessary for the production of primary metabolites required by the mycobiont for the synthesis of secondary metabolic products. These metabolites absorb radiation in the ultraviolet region [4]. One of them is an orange coloured anthraquinone pigment named parietin. It is the most common antraquinone in lichen *Xanthoria parietina*. It is deposited on the surface of mycobiont hyphae in the cortical layer of stratified lichen thalli. Parietin is highly fluorescent and occur predominantly in the apothecia, vegetative reproductive structures of *Xanthoria parietina*.

FT-Raman spectrometer with excitation wavelength 1064 nm and micro-Raman spectrometer with excitation 785 nm were used to study vibrational properties of lichen *Xanthoria parietina*. The Raman spectra taken from different part of upper surface of lichen are shown in Fig. 1. The main difference between the Raman spectra obtained from the apothecium (Fig. 1a) and from a lobe (Fig. 1b) is the absence of the bands at 1002, 1156, 1327 and 1525 cm^{-1}, which are characteristic bands of carotenes [5], in the spectrum of the apothecium. The other bands at 926, 1275, 1439, 1553 and 1672 cm^{-1} are attributed to parietin, the main secondary metabolite in *Xanthoria parietina.* It indicates that the concentration of the parietin in apothecia is higher than on the lobe.

The application of metal nanoparticles on surface of lichen *Xanthoria parietina* decreases the fluorescence from parietin and other fluorescent substances of lichen (Fig. 2). The very broad bands appearing in the SERS spectra obtained from the upper surface of *Xanthoria parietina* (Fig. 2c) suggests the presence of polymers in cell wall with the different structures and the polymerization degree. The common polymers found in the cell wall of *Xanthoria parietina* are chitin, β-glucan and galactomannoproteins [6]. After comparison of the SERS spectra of lichen with Raman spectra of chintin, β-glucan and galactomannan [7], we can conclude that in the SERS spectrum of lichen *Xanthoria parietina* are present predominantly the bands corresponding to galactomannoproteins. The SERS spectra taken from the upper and lower surface are very similar (not shown). It means that the SERS spectra of lichen taken from the upper surface, where the Raman bands of parietin are not present (Fig. 2), are due to the strong Raman signal from the cell wall constituents of lichen fungi.

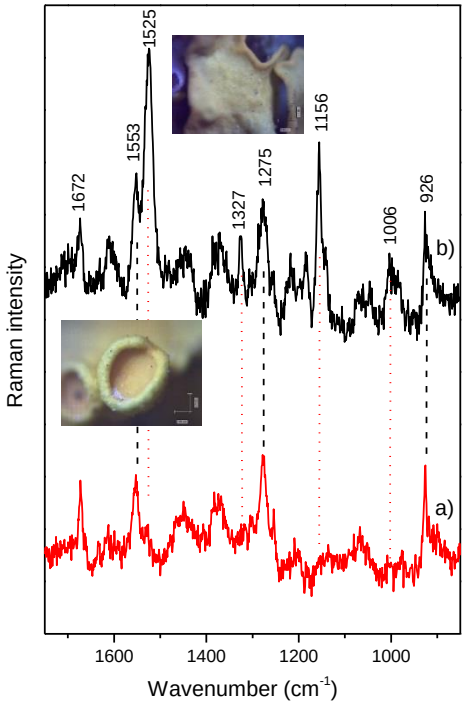

Fig. 1. Raman spectra of a) an apothecium and b) a lobe of *Xanthoria parietina*. Laser excitation 785 nm.

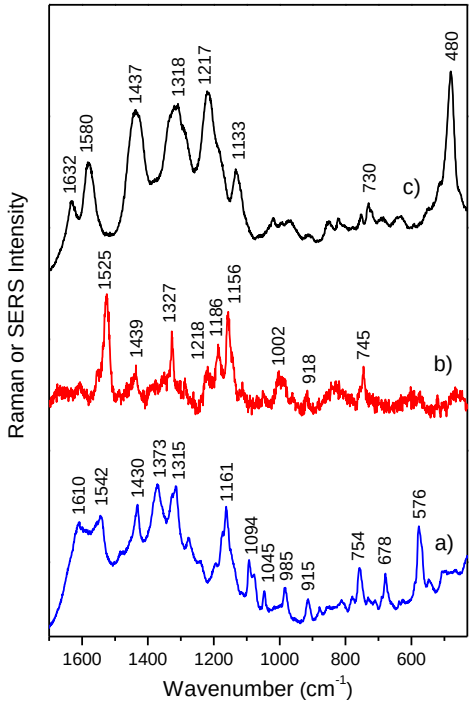

Fig. 2. a) SERS spectra of parietin, b) Raman spectra and c) SERS spectra of *Xanthoria parietina*, laser excitation 785 nm.

Altogether, this works shows possibility to combine the classical Raman spectroscopy and SERS for the detection and distribution of secondary metabolites (parietin, β-carotene) and components of the cell wall in the structure of lichen *Xanthoria parietina*.

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MEOTA-BTZ derivatives inhibit amyloid aggregation of lysozyme in linker-length dependent manner

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The formation and accumulation of amyloid aggregates is one of the accompanying phenomena of amyloidosis, which include Alzheimer's and Parkinson's diseases or non-neuropathic lysozyme systemic amyloidosis. Amyloidosis are currently untreatable and the incidence of Alzheimer's disease, the most common form of dementia, reached 47 million patients in 2016 [1]. Furthermore, this number is expected to grow by almost 10 million per year. Nowadays, an approach to the treatment of these diseases is mostly symptomatic and aimed at temporary improvement in quality of life.

One of the very promising approaches allowing to solve the problem of amyloidosis is an inhibition of amyloid aggregation of poly/peptides or removal of insoluble amyloid fibrils from the affected tissues. According to studies, small molecules have a great potential to interfere with amyloid fibrillation of poly/peptides [2, 3]. The three suggested features of compounds able to inhibit amyloid aggregation are: the presence of aromatic rings, the position of side groups on the aromatic rings and the length and flexibility of the linker between functional groups [4]. In our study, we focused on small organic heterodimers, namely tacrine-benzothiazole (MEOTA-BTZ) molecules which consist of 7-methoxytacrine and 2-aminobenzothiazole functional groups linked by an alkylamine linker.

Using *in vitro* and *in silico* methods we investigated the ability of the studied compounds to significantly inhibit the amyloid aggregation of hen egg white lysozyme (HEWL). The effect of compounds was quantitated using thioflavin T (ThT) fluorescence assay. The inhibitory effect was observed in the case of all compounds with IC_{50} values in the micromolar range; however, the effectivity of compounds differed based on length of the linker. Measured data correlate with images from atomic force microscopy. The most effective derivative contains the longest, eight carbon long linker. These observations were supported by *in silico* calculations suggesting the direct dependence between the binding free energy of the derivatives and the length of the linker. MEOTA-BTZ derivatives have convincingly demonstrated their ability to inhibit the amyloid aggregation of HEWL at tolerable cytotoxicity level.

Acknowledgement
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Application of induced CD signals for identification of G-quadruplexes in coding and noncoding regions in HIV sequences

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Recently, the presence of G-quadruplexes (G4) in various viruses and their involvement in key steps of virus life cycle has been reported. G4 have been implicated in pathogenic mechanisms of the Epstein-Barr virus, SARS coronavirus, herpes simplex virus 1 and the human papillomavirus. Functionally, significant G4 have been identified in the Nef coding region and in the unique long terminal repeat (LTR) promoter of the human immunodeficiency virus (HIV). HIV is one of the most studied viruses in the world and the etiologic agent of the acquired immune deficiency syndrome (AIDS), a major worldwide epidemic [1].

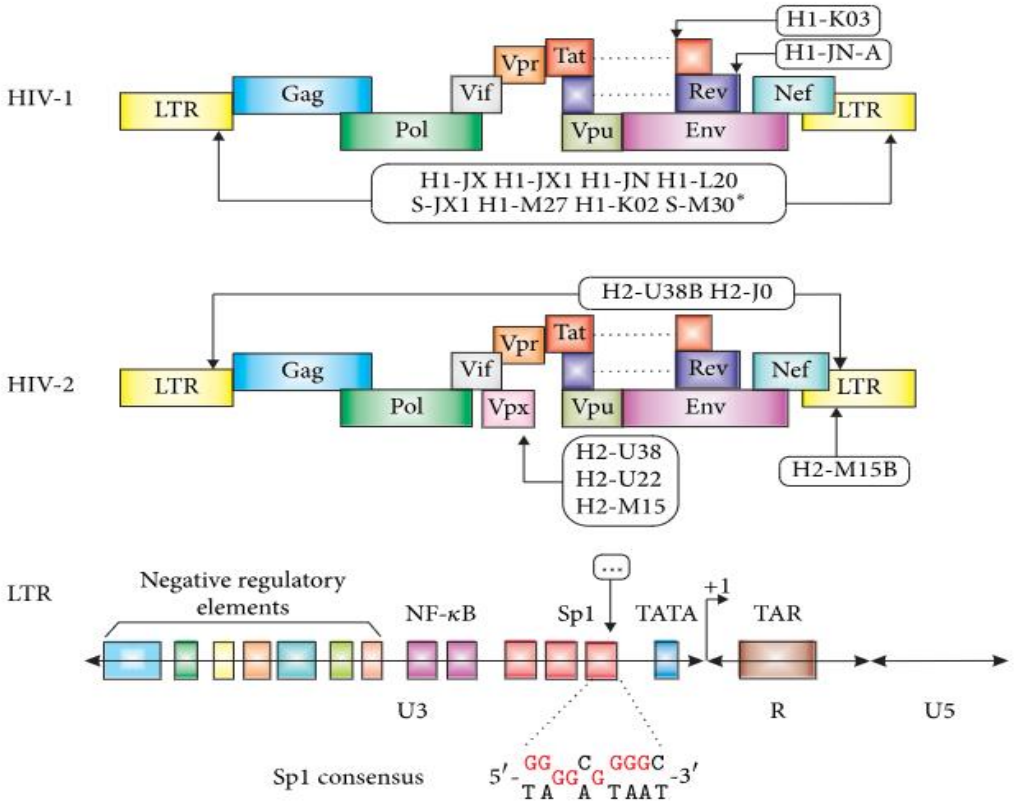

Fig. 1. Schematic representation of HIV-1 and HIV-2 genome organizations and locations of studied sequence [2].

More than 9 thousand genomic sequences of HIV isolates have been sequenced and analyzed. We have analyzed series of DNA sequences with the potential to form G4 structures. Several such sequences were found in various coding and noncoding virus domains, including the U3 LTR, tat, rev, env, and vpx regions [2]. These domains are not necessarily located only in regulating LTRs, but also in other gene coding regions. In addition, G-rich domains were also located in the minus-strand of many HIV-1 isolates, the sequence of which is highly homological with the well-known sequence

forming the interlocked and extremely stable HIV integrase aptamer [3]. The sequences derived from original isolates were analyzed using standard spectral and electrophoretic methods. In addition, a new strategy developed in our laboratory where the unique spectral profile of G4-Thiazole Orange (TO) complexes of induced circular dichroism (ICD) was applied [2]. The ICD of any such complexes shows the positive signals at ~495 and ~510 nm, and the negative signals at ~475 nm at certain condition. TO stabilizes G-quadruplex structures, it may also induce topological conversion of antiparallel to parallel form and facilitates G-quadruplex multimerization. The ICD signatures can be used to determine whether specific unknown sequences form G-quadruplex motifs [4].

Targeting the G4 or peptide domains corresponding to the G-rich coding sequence in HIV offers to researchers attractive therapeutic targets which would be of particular use in the development of novel antiviral therapies. The analysis of G-rich regions can provide researchers with a path to find specific targets which could be of interest for specific types of virus [2].

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Calcium mediated DNA complexation with zwitterionic and anionic liposomes

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Gene therapy has a great potential for treatment of many inherited and acquired diseases. For gene delivery into cell a suitable vector is needed. Nonviral vectors, such as cationic liposomes and polymers, form complexes with DNA that facilitate cellular uptake and delivery. However, cationic liposomes show high cytotoxicity both *in vitro* and *in vivo* [1,2]. This problem may be partially overcome by substituting the synthetic cationic lipids by endogenously occurring anionic or zwitterionic lipids [3,4]. The positive charge needed for a formation of lipoplexes is in this case provided by divalent metal ions (Ca^{2+}, Mg^{2+}, etc.) from which calcium proves to be most efficient [5]. The anionic lipoplexes composed of a ternary complex of endogenous occurring non-toxic anionic lipids and plasmid DNA using divalent Ca^{2+} bridges has high transfection efficiency comparable with cationic lipoplexes, but with significantly lower toxicity [3,4].

In present work we studied the ability of anionic and/or zwitterionic liposomes to form complexes with DNA in the presence of Calcium ions. Formation of complexes as a function of Ca^{2+} concentration was examined employing UV-Vis spectroscopy by determination of the unbound DNA fraction in the sample. We also examined the microstructure of formed complexes using synchrotron small-angle X-ray diffraction (SAXD). The studied liposomes were composed of zwitterionic lipid 1-palmitoyl-2-oleoylphosphatidylethanolamine (POPE) and anionic lipid 1-palmitoyl-2-oleoylphosphatidylglycerol (POPG) or the pure POPE respectively.

The lipid mixture was prepared by mixing of lipids in methanol, and consecutively, organic solvent was evaporated by nitrogen gas and under vacuum. The complexes were prepared by hydration with the POPE/POPG = 9:1 mol/mol mixture or the pure POPE with DNA and $CaCl_2$ dissolved in 0.15 mol/l NaCl solution. Small-angle (SAXD) synchrotron X-ray diffraction experiments were performed at the soft condensed matter beamline A2 at HASYLAB, DESY.

Ca^{2+} ions mediate the interaction between DNA and anionic or zwitterionic liposomes and enable formation of lipoplexes. The interaction is electrostatic. The formation of complexes with DNA for both POPE and POPE/POPG liposomes showed dependence on Ca^{2+} concentration. The higher efficiency to bind DNA was reached by POPE liposomes which bound over 98% of the DNA already at 2.5 mmol/l of Ca^{2+}. The POPE/PEPG liposomes reached the similar DNA binding at the concentration of Ca^{2+} 15 mmol/l. The SAXD showed the organization of lipid in a lamellar phase. In all samples we observed a lamellar phase assigned to pure lipid and based on the Ca^{2+} concentration we also observed the condensed lamellar phase of DNA-liposome complexes. For POPE we observed small peak assigned to condensed lamellar phase already at 1 mmol/l of Ca^{2+}. With increased concentration of Ca^{2+} to 2.5 mmol/l the fraction of condensed lamellar phase was significantly increased and with further increase of the Ca^{2+} concentration remains more or less unchanged. With POPE/POPG liposomes we observed the presence of condensed lamellar phase only above the concentration of Ca^{2+} 10 mmol/l with maximal fraction of condensed lamellar phase to lamellar phase of pure lipid reached at 15 mmol/l of Ca^{2+}.

In a conclusion both studied liposomal systems showed good ability to form complexes with DNA in the presence of Calcium. The effectiveness to form complexes was significantly higher for zwitterionic liposomes formed by pure POPE, while the anionic POPE/POPG liposomes needed higher Ca^{2+} concentrations to reach similar binding of DNA.

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Lipid-mediated signaling in cancer cells

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Lipids play an important role in signaling by activation of lipid-regulated kinases as protein kinase C (PKC). We have focused our interest on lipids and especially low-density lipoprotein (LDL) activation of PKC signaling in aggressive types of cancers. We have previously shown that U87 MG glioma cells express a high number of LDL receptors [1] that allow increasing of LDL uptake by these cells [2, 3]. Two PKC isoforms (PKCα and PKCδ) were also recognized in U87 MG cells [4, 5]. In present work, we have identified PKCα and PKCδ levels in two breast cancer HER2 positive cell lines.

Western blotting analysis revealed higher LDL receptors and PKCδ isoform in SK BR 3 cells but low level of both in BT 474 cells. We have observed that PKC regulators (PMA, hypericin, rottlerin) affected the levels of LDL receptors in all studied cell lines. While PMA decreased LDL receptors and increased PKCδ levels, cell treatments with rottlerin and hypericin resulted in LDL receptors increase and PKCδ decrease. However, these effects could be amplified or reduced by lipids presence/absence in the cell culture medium. We have shown that LDL importantly influenced PKCδ activation.

LDL uptake is governed by the endocytotic pathway. Subcellular organelles that are affected by lipid metabolism are lysosomes and Golgi apparatus. We have demonstrated by confocal fluorescence microscopy and fluorescence lifetime imaging that the regulation of PKCδ is accompanied by alteration of Golgi apparatus and the numbers of lysosomes. These alterations finally resulted in modification of endocytosis and LDL uptake by cancer cells. We hypothesize that the fluidity of the membrane was importantly changed.

In summary, we have demonstrated that lipids can up-regulate but also down-regulate PKCδ signaling in aggressive types of cancer cells.

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Direct synthesis of metal nanoparticles by intense laser fields in aqueous solution

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In this contribution, we implemented a new method for synthesis of nanoparticles (NP) by ablation of metal targets by femtosecond laser pulses in pure water. This pathway of photochemical synthesis allows to produce NP from metallic or non-metallic precursors in various transparent media e.g. emulsions, solvents, glasses, polymers without the need of any chemical add-ons.

NP's synthesized from Co, Zn and Ni targets in pure water were characterized by UV-VIS absorption spectroscopy, scanning electron microscopy (SEM) and confocal laser scanning microscopy (CLSM) imaging with spectral detection. NP solution dried on silicon substrate imaged by SEM microscopy is shown at Fig.1. Shape of NP was generally spherical and their size range was approximately 0–20nm for Co and Zn NP's, and 10–100 nm for Ni particles.

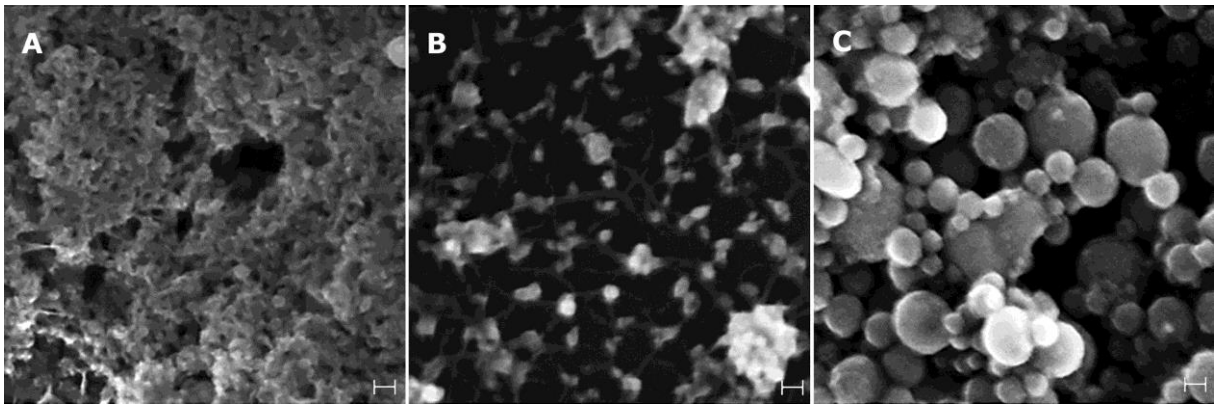

Fig. 1. SEM imaging of metal nanoparticles. A) Cobalt, B) Zinc, C) Nickel. Scalebar: 20nm

Absorption spectra of metal NP's colloids in water are shown at Fig.2A. The data were normalized to the absorbance measured at 300nm, ratio of absorbance at this wavelength were 2 | 3 | 1 for Co | Zn | Ni, respectively. NP samples of Cobalt and especially Zinc show strong plasmon resonance peak at UV wavelengths (390nm for Co and 350nm for Zn). In comparison, absorbance of nickel NPs has no pronounced spectral features. These results indicate correlation of NP size / composition and their plasmonic resonance properties [1,2].

The ability of NP's to generate fluorescent signal is an interesting phenomenon with potential of future applications for sensing purposes. Spectrally-resolved imaging of the Zn NP deposits upon drying on silicon wafer (Fig.2B) show distinct green fluorescence of Zinc NP peaking at 520nm (ROI 1), compared to the non-specific background signal from silicon substrate (ROI 2). Origins of fluorescence signal generated by metal NP is still not successfully described [3,4] and demands future investigation.

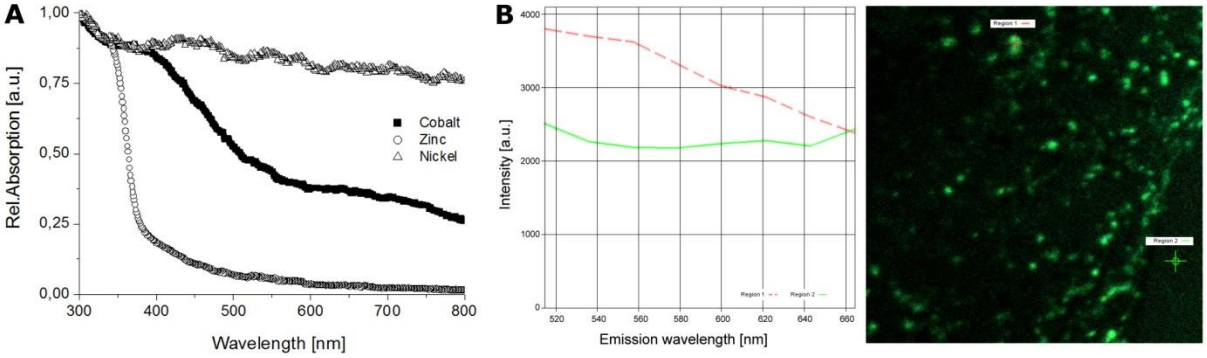

Fig. 2. A) Absorption spectra of NP colloids. B) Spectrally resolved image of Zn nanoparticles dried from water solution at Si substrate, with two spectral profiles measured at selected region of interests (ROI 1,2).

In summary, we successfully designed and created a novel apparatus for generation of metal NP in aqueous solution from potentially any type of target. Presented results are the first steps towards on-demand synthesis of metal NP with potential applications in various fields of biomedical and environmental sciences.

Acknowledgement
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Modulated photophysics of curcumin entrapped into poly(2-oxazoline) based micellar assembly: The influence of molecular weight of lipophilic part on the formulation

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Polymeric micelles have been extensively used for the targeted delivery of anticancer drugs to tumor sites by passive as well as active mechanism. Present study provides important information on the nano-formulation of curcumin, a bioactive compound using poly(2-oxazoline) which are considered as the new generation polymer therapeutics. Here we have investigated the role of the hydrophobic block of 2-oxazoline based gradient copolymers on the modulation of the photophysical properties of curcumin trapped inside the micellar assembly. The solubility and chemical stability of curcumin is largely enhanced due to encapsulation inside the micelle and it depends very much on the hydrophobicity of core of the polymeric micelle. The fluorescence quantum yield is also enhanced along with the blue shifting of the emission maxima which is a consequence of the reduced interaction of curcumin with water. The rate of the major excited state deactivation pathway of curcumin through intramolecular proton transfer process is retarded due to the intermolecular hydrogen bonding with micellar core.

Acknowledgement
The authors are thankful to the Slovak Grant Agency VEGA for the financial support in the project No.2/0124/18 and the Slovak Research and Development Agency for financial support in the project No. APVV-15-0485.

Spectroscopic (Raman and IR) characterization of the neuropeptide Substance P structure

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Substance P (SP; Fig. 1), a positively charged 11-amino acids neurotransmitter peptide (Figure 1), is a part of tachykinins, one of the largest family of neuropeptides. It is widely distributed in both the central and peripheral nervous systems and in gastrointestinal tissues. This hormone is an important element in pain perception, but it is also involved in modulating immune response, inflammation, blood flow, and in promoting wound healing of non-healing ulcers [1-3]. All these roles stimulate the interest of pharmaceutical industry in the design and synthesis of SP agonists and antagonists because of their potential therapeutic applications in the treatment of a variety of stress-related illnesses and use as analgesics [2]. Thus, understanding the relationship between the structure and biological activity would assist in developing therapeutically valuable SP analogues or new peptidomimetics. In this context, vibrational spectroscopy (Raman and IR) can give a useful contribution since it can define small regions in large macromolecular complexes [4], and gives information on some specific functional moieties (such as –SH, -SCH_3, *etc.*) [5, 6]. Moreover, it can also give a global insight into the overall secondary structure of proteins [7, 8].

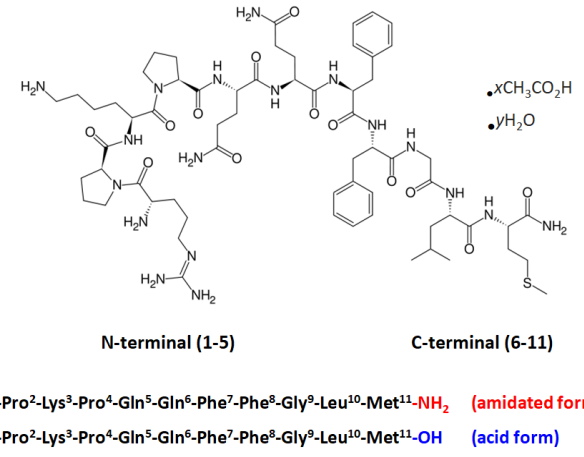

Fig. 1. Amino acid sequence of SP

The present work is focused on the vibrational analysis of SP under different experimental conditions in order to obtain a deeper insight into its general structural properties. In particular, Raman spectra of SP in the amidated form were recorded at different pH whereas the Raman Amide I bands were analyzed by computer curve fitting. It was shown that a higher orderliness in the SP secondary structure is induced by the pH increase (Figure 2A) what agrees with the capability of SP to self-assembly and form aggregates [9]. The presence of the latter was also confirmed by analyzing the corresponding IR spectra.

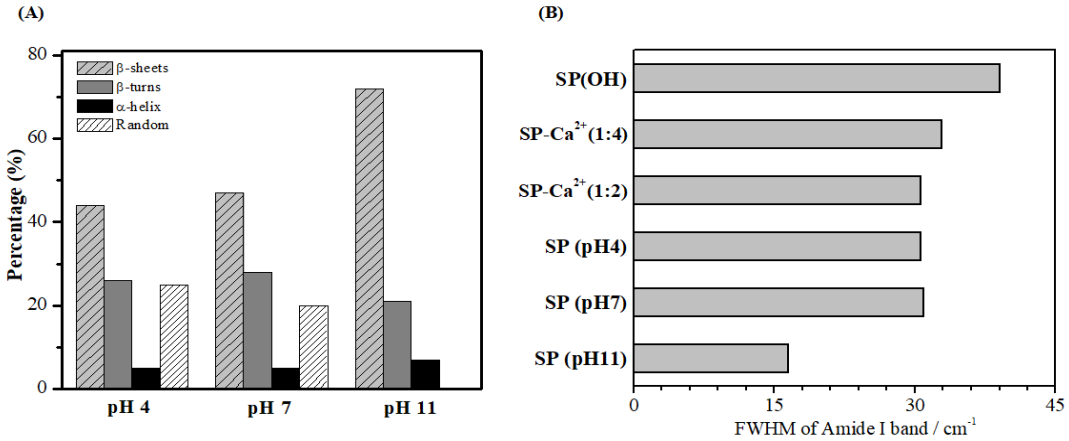

Fig. 2. (A) Block diagram of the percentages of the secondary structures found in SP at different pH, calculated by the curve fitting analysis of the Raman Amide I regions. **(B)** Full width at half maximum (FWHM (cm^{-1})) of the Raman Amide I bands of the SP amidated form at different pH; in presence of Ca^{2+} ions, and in acidic form (SP(OH)) at pH 7.

It is well known, that SP encounters millimolar concentrations of Ca^{2+} in the extracellular compartment what, in addition, seems to be a necessary factor for the SP action [10]. Therefore, the effects of interactions with Ca^{2+} ions to the SP folding were also evaluated (Figure 2B). The interaction of SP with the metal ions (1:4 peptide/Ca^{2+} molar ratio) is able to induce structural changes in SP, such as a decrease in the β-sheet content and an increase of the α-helical structure. This can be connected with the literature reporting that Ca^{2+} ions may bind to the peptide hormone and impose additional structural constraints [11].

Finally, the capability of Surface-enhanced Raman spectroscopy (SERS) to detect SP in its biologically active form (amidated form) at trace concentrations was also tested. It is possible only at neutral pH thanks in particular to the interactions between surface metal atoms and the very active $–SCH_3$ group of Met.

Acknowledgement
This work is supported by the Scientific Grant Agencies of the Ministry of the Education of Slovak Republic (VEGA 1/0929/16 and APVV-15-0485).

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Non-lamellar lipid mesophases in drug delivery

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Gene therapy as a strategy for treatment of various disorders such as cancer, cardiovascular diseases or congenital disorders arises now. Delivery of genetic material (DNA) into cells is achieved via vectors with viral or nonviral origin.

Viral vectors present good transfection efficiency, but there is a problem with their possible immunogenic, proinflammatory and carcinogenic activity [1].
Nonviral vectors have low toxicity and antigenicity, because they are formed of biocompatible particles, although their transfection efficiency is lower.

Positively charged cationic lipids interact with negatively charged molecule of DNA to form complexes known as lipoplexes. There is a possibility to prepare lipoplexes composed of neutral or anionic lipids combined with divalent cations. Divalent cations such as Ca^{2+} mediate complexation between DNA and the anionic lipid [2].

The study examines polymorphism of lipid mixtures without and with presence of calcium cations. Neutral MON (1-monooleyl-rac-glycerol) and anionic DOPG (1,2-dioleyl-sn--glycero-3-phosphatidylglycerol) were mixed at different molar ratios. Samples were hydrated with aqueous solution of 150 mM NaCl. The concentration of calcium ions was 14,7 mM. Short fragmented double strain DNA from salmon sperm were used for the lipoplexes preparation. The structure of mixtures was examined by polarised light microscopy and small angle X-ray diffraction (SAXD).

A cubic phase Pn3m was detected in mixtures MON-DOPG with ≤ 10,8 mol % DOPG. The structural parameter of Pn3m phase increases with increasing of DOPG content, and consecutively, the pattern from SAXD disappeared at high mol % DOPG. Increased content of DOPG disintegrates cubic phase and support formation of a lamellar phase. Microscopy shows patterns typical for multilamellar structure (Fig. 1) even if SAXD didn´t confirm its presence.

We observed the coexistence of two cubic phases, Pn3m and Im3m, when Ca^{2+} cations were added to MON-DOPG mixtures containing ≤ 31,8 mol % DOPG. Calcium ions neutralise anionic DOPG which gave the possibility of the formation of cubic phase also at higher content of DOPG, as shown in [3].

DNA addition affected structural polymorphism. Three phases, cubic phase Pn3m, Im3m and an inversed hexagonal phase H_{II} were identified in MON-DOPG-Ca^{2+}-DNA aggregates (Fig. 2) containing ≤ 37,2 mol % DOPG. In the presence of DNA, the structural parameter of cubic phases changed towards lower values (Fig. 3).

The amount of DNA complexed in aggregates was examined by UV-VIS spectroscopy. Although the highest content of complexed DNA was found in MON-DOPG-Ca^{2+}-DNA aggregates with 77,2 mol % DOPG (Fig. 4), the structure of the complex remained unknown due to the absence of SAXD pattern. At 16,4 mol % of DOPG, the binding capacity of DNA was relatively high and represent 20,3 % complexed DNA. The SAXD pattern confirmed superposition of Pn3m and Im3m cubic phase. With the appearance of an inversed hexagonal phase, the DNA binding rapidly decreases, then slowly raised up to 15,7 % DNA.

To conclude, the structural polymorphism was affected not only by the lipid composition and interaction with Ca^{2+}, but also by the addition of DNA. DNA induced a

formation of an inversed hexagonal phase. The best binding capacity for DNA we detected in aggregates with cubic phases.

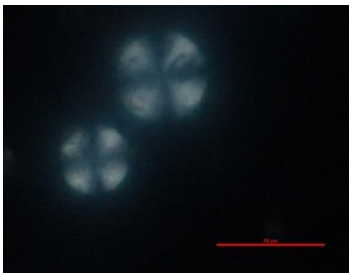

Fig.1. Maltese cross pattern from polarized light microscopy suggests presence of multilamellar vesicles in MON-DOPG mixtures at 21,5 ≤ mol % DOPG ≤ 100. Scale in red 50 μm.

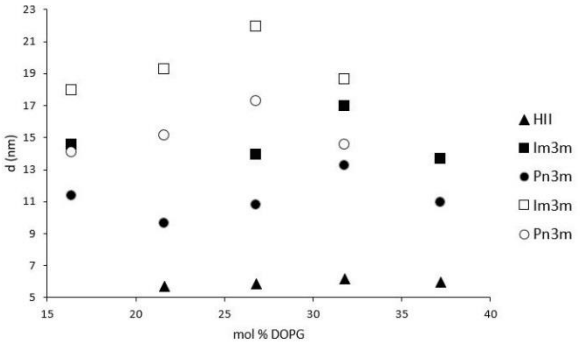

Fig.3. Structural parameters for MON-DOPG-Ca^{2+} (white) and MON-DOPG-Ca^{2+}-DNA (black) complexes.

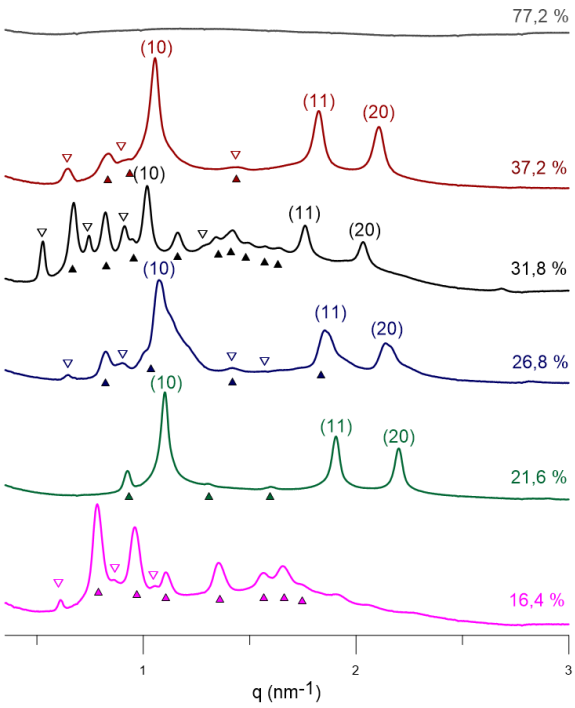

Fig.2. SAXD pattern of MON-DOPG-Ca^{2+}-DNA aggregates. Content of DOPG shown as mol % DOPG. ▲ Peaks of Pn3m phase ▽ peaks of Im3m phase. For H_{II} phase Miller indices are marked. Intensity is in logarithmic scale.

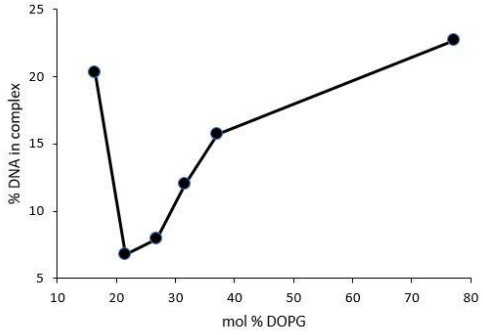

Fig.4. DNA binding in MON-DOPG-Ca^{2+}-DNA aggregates as a function of DOPG content.

Acknowledgement
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Optically trapped micro-structures prepared by two-photon polymerisation

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Optical tweezers (OTs) technique, due to its unique non-contact and non-invasive characteristics [1] is widely used in many areas of science, above all for trapping, transporting and manipulation of micro-structures or nano- structures. The trapping ability of the OTs is limited with target objects' size, shape, material, index of refraction, etc. Many experiments require particles of non-spherical shape and arbitrary size. Photo-polymerization techniques evolved during the last two decades and are now available for direct manufacturing of solid micro-objects with spatial details in the order of 100-200 nm [2].

In this work, we demonstrate fabrication, on-demand release, and optical manipulation of the micro-structures prepared by photo-polymerization. We also propose manipulation ability extension of the optical tweezers by developing micro-hands, which are to be used as end-effectors of the laser beam.

First, different 3D structures with micro-scale handles are designed and fabricated on a glass substrate by the two-photon polymerization method. Next, the prepared structures are released from the substrate and are transferred into a sample cell (to be used under OTs) by means of micropipettes mounted on nano-manipulators. The released structures are manipulated by holographic multiple-spot optical tweezers (HOT), which traps and manipulates numerous objects simultaneously. Where direct trapping of the target object is not possible, indirect manipulation of the target micro-objects is achieved by using the micro-hands as an extension of optical tweezers.

All these works indicate that OTs system is a very useful tool that has great potentials in life sciences and nano-sciences. Image processing for automation of indirect manipulation tasks will be investigated as a future work.

Acknowledgement
This work was supported by the research grant from the Slovak Grant Agency Vega No. 2/0086/16 and APVV 15/0665.

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Influence of LDL ageing on hypericin delivery into cancer cells

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Low density-lipoproteins (LDL) are the group lipoproteins that major role is to transport cholesterol into the cells. These important molecules participate in the transportation of hydrophobic molecules from extracellular environment into the cells. Such principle was adopted in a delivery of hydrophobic photosensitizers into the cancer cells in the photodynamic therapy (PDT) [1]. Photodynamic therapy comprises three main compounds: oxygen, light and photosensitizer that is activated by light at appropriated wavelength. In our study we used hypericin (Hyp) as a model photosensitizer.

Hyp is a naturally occurring photosensitizer found in the plant common St. John's Wort (Hypericum species). It is a well-known therapeutic and diagnostic agent in cancer detection and treatment. High hydrophobicity is a characteristic feature of Hyp. Due to this property it is insoluble in water [2].

In the present study, we have monitored Hyp distribution and delivery in the glioma cancer cells and two different breast cancer cell lines. Hyp was delivered in LDL that was affected or not by ageing: LDL_{old}/Hyp and LDL_{fresh}/Hyp, respectively. Our aim was to identify the rate of difference between Hyp delivery and intracellular distribution by fresh and old LDL.

Spectroscopic properties between fresh and old LDL were assessed by absorption and fluorescence spectroscopy and SERS-Raman spectroscopy approach. We have demonstrated that fresh 10 nM LDL could be loaded by monomeric Hyp up to a ratio 1:50 Hyp in LDL, but in old 10 nM LDL was this ratio up to 1:20.

Endocytosis of LDL is mediated by LDL receptors that are expressed in certain types of cancer cells. We have evaluated LDL receptors expression in U87 MG, SK-BR-3 and BT 474 cells. No significant difference in LDL receptors expression was observed after 1 h incubation of cells with fresh and old LDL.

Hyp uptake by U87 MG, SK-BR-3 and BT 474 cells was observed by confocal fluorescence microscopy. Clear differences in Hyp intracellular distribution and concentration was recognized. While fresh LDL kept Hyp in the complex (Hyp:LDL) and persist in lysosomes, Hyp easily released from old LDL and further, Hyp was homogenously distributed in cells.

In further, the distribution of Dil labeled LDL and Dil labeled acetylated LDL was monitored by confocal fluorescence microscopy. Hyp delivery by these two systems (Dil LDL and acetylated Dil LDL) recognized lysosomal localization of Hyp when Dil LDL were used and homogenous Hyp distribution when acetylated Dil LDL were incubated with cells. We have found similarities between old LDL and acetylated LDL Hyp uptake and Hy distribution.

Our findings show, that Hyp distribution in the complex with old LDL/acetylated LDL is the same. If Hyp is in the complex with fresh LDL, Hyp is delivered *via* endocytotic pathway.

In summary, we have demonstrated significant differences between Hyp distribution and delivery in the complex with LDL_{fresh} and LDL_{old}.

Acknowledgement
This work was supported by the research grant from the Slovak Grant Agency Vega No. VEGA 1/0156/18 and APVV-15-0485.

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Unexpected dual effect of non-ionic detergent Triton X-100 on insulin amyloid formation

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Atomic force microscopy, Thioflavin T (ThT) fluorescence assay, circular dichroism spectroscopy, differential scanning calorimetry, and computer simulation analysis have been employed to investigate the amyloid aggregation of insulin in the presence of non-ionic detergent, Triton X-100 (TX-100). In contrast to recently described inhibition of lysozyme amyloid formation by non-ionic detergents [1], the amyloid aggregation of insulin in the presence of sub-micellar TX-100 concentration exhibits two dissimilar phases. The first, inhibition phase, is observed at the protein to detergent molar ratio of 1:0.1 to 1:1. During this phase, the insulin amyloid fibril formation is inhibited by TX-100 up to ~60%. The second, "morphological" phase, is observed at increasing detergent concentration, corresponding to protein/detergent molar ratio of 1:1 - 1:10. Under these conditions a significant increase of the steady-state ThT fluorescence intensities and a dramatically changed morphology of the insulin fibrils were observed. Increasing of the detergent concentration above the CMC led to complete inhibition of amyloidogenesis. Analysis of the experimental results as well as computer simulation analysis suggests the existence of two TX-100 binding sites on insulin.

Acknowledgement
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Cell morphology and metabolism reflect the physiological state of cells

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Mitochondrial function and its defects underlie many of civilization diseases such as Parkinson's and Alzheimer´s diseases, cardiovascular pathologies or cancer. In our study, we have focused on electron transport chain (ETC) in U87 MG cells. We have used rotenone, a ETC complex I inhibitor, to induce mitochondrial dysfunction in cells similar to Parkinson disease. Using confocal fluorescence microscopy, we have characterized the changes in mitochondria morphology and function. Complex I impairment leads to compromised cell respiration, loss of mitochondrial membrane potential, and generation of free radicals such as superoxide anion and hydrogen peroxide.

To alleviate the rotenone effects, we have used photobiostimulation (PBM) with low level near infrared (NIR) light. The use of low level of visible or near infrared (NIR) light can be used to reduce pain, inflammation, support the treatment of injuries, and prevent tissue damage (Fig. 1). Mitochondria are the site of initial light exposure, ROS modulation, induction of transcription factors.

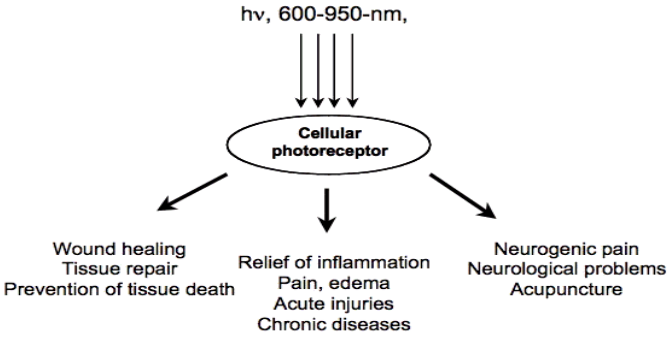

Fig. 1. Potential PBM effects.

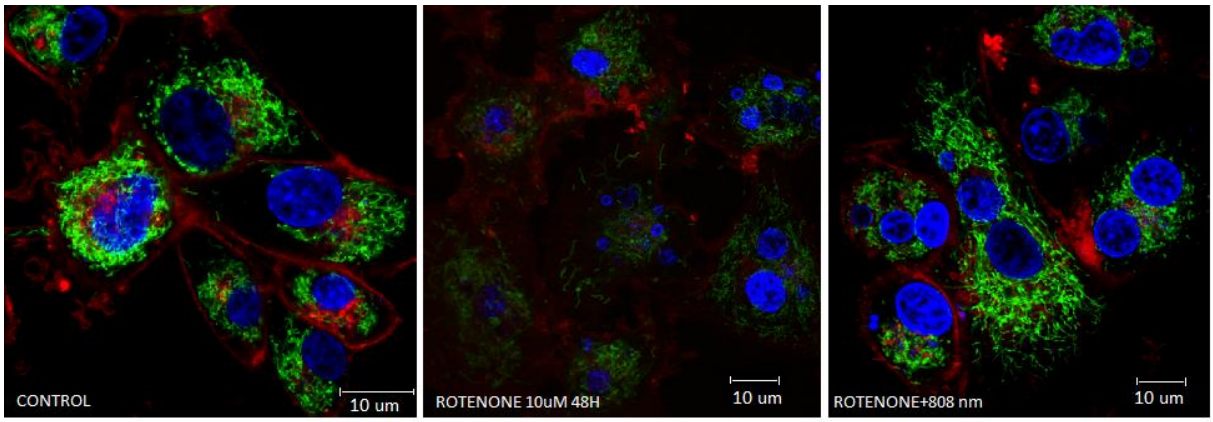

Fig. 2. PBM effect on cell morphology.

Impaiment in U87 MG cells was induced with rotenone. Rotenone administration resulted inchanges in mitochondria, nucleus and plasma membrane. We expect that morphological changes in mitochondria are linked with their altered function. PBM treatment

induced mitochondrial fusion and improved damaged mitochondria function (Fig. 2). In conclusion, the PBM treatment can improve damaged cells function, however more studies regarding the dosimetry and molecular mechanisms underlying PBM effects are necessary.

Acknowledgement
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Cytotoxic effect of morphologically different types of lysozyme amyloid structures on SH-SY5Ycell line.

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For several amyloid diseases, including Alzheimer´s and Parkinson´s diseases, lysozyme systematic amyloidosis and diabetes mellitus, the conversion of soluble protein into amyloid structures plays the key role. The fact that amyloid aggregates display common features suggest that formation of amyloid aggregates is a generic property of all poly/peptides. It is generally accepted that protein fibrillization is a result of intermolecular interactions between non-native protein conformers leading to formation of amyloid nuclei required for their further oligomerization and elongation into mature amyloid fibrils [1]. Amyloid aggregates accumulate in extra or intracellular space into amyloid deposits (plaques, tangles) leading to cytotoxicity.

Amyloid aggregates can be divided to two main groups: i) oligomeric species and ii) fibrillary structures. Oligomeric species are small (~3-10 nm), spherical structures while the mature amyloid fibrils are highly ordered and stable linear protein filaments (3-10 nm in diameter) [2]; their core structure is characteristic of cross β-sheet structure including β strands that are perpendicular and parallel to fiber axis. The final structure of amyloid aggregates can be affected by temperature, pH, protein concentration and presence of salts [3]. It was found that structurally different aggregates could induce different mechanisms of toxicity to neuronal cells [4]. Recently, it was generally accepted that the most toxic amyloid species are early soluble oligomeric structures, and mature amyloids fibrils are mostly considered less toxic [5-6].

In the present work, we have studied relationship between the morphology of amyloid aggregates of hen egg white (HEW) lysozyme and their cytotoxicity. We have investigated the effect of morphologically different amyloid structures (oligomers, protofibrils, fibrils) on the proliferation and viability of neuroblastoma SH-SY5Y cell line. The HEW lysozyme amyloid aggregates structures were characterized using Thioflavin T fluorescence spectroscopy and atomic force microscopy. The proliferation and viability of neuroblastoma cells was observed using cell proliferation assay WST-1. We have found that different lysozyme amyloid structures show different effect on proliferation activity of neuroblastoma cells. The data obtained after 24h and 48h incubation of SH-SY5Y cells with lysozyme aggregates suggest that cell proliferation activity and viability significantly decreased for cells treated with oligomeric structures. The obtained results indicate that oligomeric aggregates have the most significant cytotoxic impact on the neuronal cells.

Acknowledgement
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Studies on the azo dyes aggregation and their binding to albumin

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The azo dyes represents class of organic compounds composed of aromatic rings, which are linked through the azo group. The majority of azo dyes are water-soluble, and their application is very wide; from dyeing textile fibers to staining biological samples. One of the most common azo dye - Evans blue (EB) is known to bind serum albumins [1]. Albumin-bound EB is frequently used in estimating blood volume and vascular permeability, detecting lymph nodes, localizing the tumor lesions or estimating permeability of blood-brain barrier [2, 3]. Structurally similar dye Congo red (CR) is used as a cytoplasm and erythrocyte stain or as amyloid specific dye for *in situ* staining and *in vitro* detection of amyloids [4]. Unlike other dyes, EB and CR are able to form ordered self-associated supramolecular structures in water solutions [4-7]. The individual dye molecules are organized into ribbon-like aggregates, which possess altered binding properties in respect to proteins when compared to monomers. Several assembled dye molecules can interact with protein as a single ligand [7]. This ribbon-like structure has to be taken into account especially for quantitative analysis of dye-protein binding.

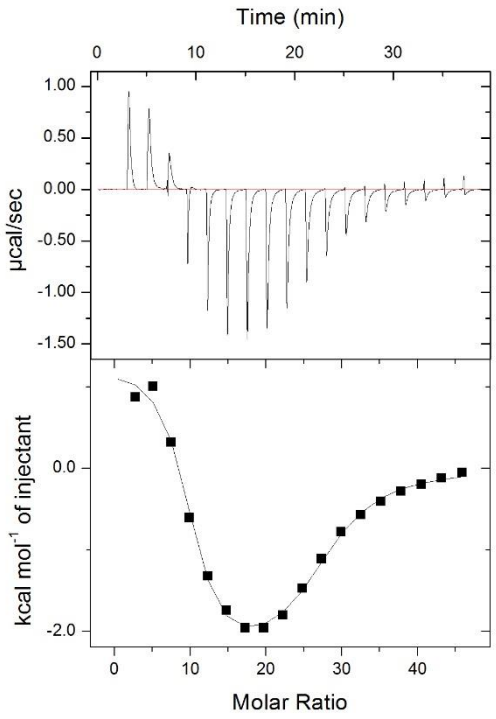

Fig.1. ITC profiles of the interaction of CR (19 x 2 µl aliquots of 7 mM) and BSA (30 µM in cell). Top panel: raw data of CR titration to BSA in 10 mM posphate, 140 mM NaCl, pH 7.4, 20°C. Bottom panel: integrated heat results of the titration with the best fitted curves to the „two set of binding sites" model.

In this work we have studied complexation of bovine serum albumin (BSA) with EB and CR using isothermal titration calorimetry (ITC), dynamic light scattering (DLS) and 2D DOSY NMR spectroscopy. DLS and NMR measurements confirmed formation of EB aggregates depending on dye concentration (0.01- 4 mM) and presence of salt (0 – 140 mM NaCl), which modulates dye-dye interaction by shielding charged groups. From ITC measurement follows, that both dyes form aggregates since the dilution of dyes during titrations is endothermic process. The interaction of EB molecules is enhanced by the presence of salts, for CR this effect is less prominent. Moreover, from the dependence of dilution enthalpy *vs.* ligand concentration is evident the aggregates are ribbon-like rather than micellar since the dependence is exponential but not sigmoidal in wide concentration interval.

The binding of either dye to BSA is complex biphasic process (Fig.1). The best fitted parameters correspond to two set of binding sites model. The first binding event is characterized by two order of magnitude higher binding affinity comparing to second event. The stoichiometric ratio N corresponding to first event is lower than that for second event (e.g. 3.8

vs. 13.6 for CR in 10 mM phosphate buffer) for both dyes and slightly increases with the salt concentration. For the interpretation of obtained N values especially taking into account the aggregated state of dyes able to bound into single binding site or the co-dependence of two binding sites the additional results including *in silico* modeling is required.

We believe that our results will help to understand the mechanism of binding of self-aggregating dyes as well to test the potential utilization of albumin as a carrier of self-assembling drugs.

Acknowledgement

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Potentiometry as a tool to study enzyme kinetics

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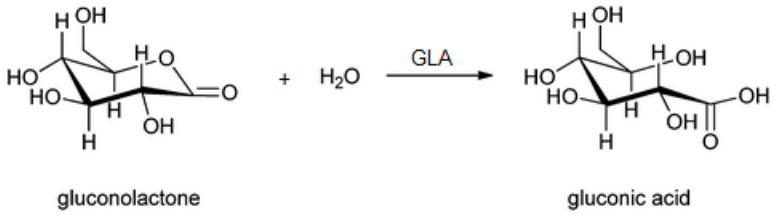
Generally, correct determination of a rate of any enzyme-mediated reaction is necessary for enzyme activity and thus its purity calculation. Most of the methods rely on the optical changes in the solutions containing either consumed substrate or forming product. The problem would arise if neither substrate nor product produces a signal in UV-Vis spectral range (they are the so-called 'optically silent' compounds).

Gluconolactonase (GLA, EC 3.1.1.17) is a hydrolytic enzyme that participates in pentose phosphate pathways, ascorbate and aldarate metabolism, and in a caprolactam degradation. The enzyme catalyzes hydrolysis of δ-gluconolactone to D-gluconic acid according to the following scheme:



Both, the substrate and the product of the reaction, are in fact 'optically silent'. Up to now, discontinuous (fixed-time) methods have been used to determine reaction rate of gluconolactonase-catalyzed reaction [1,2]. We have developed a continuous procedure that uses an electrochemical technique – potentiometry. The method is based on the fact that during the course of the reaction D-gluconic acid (a weak acid) dissociates and released hydrogen ions are detected by a sensitive glass membrane pH electrode (Figure 1A). The original pH versus time plot has been transformed to $[H^+]$ on time dependence (Figure 1B) and the polynomial function has been used to fit the data. Finally, calculation of the first derivative of [H+] with respect to time give us information on the reaction rate at corresponding pH value (Figure 1C).

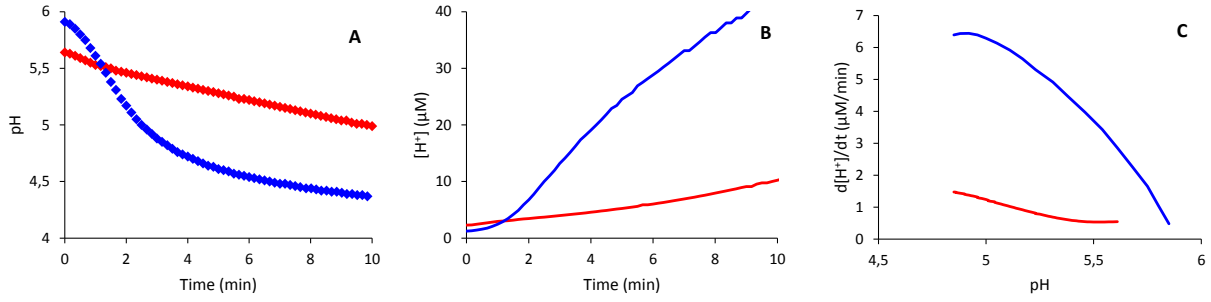


Fig. 1. (A) Time-dependent pH change during δ-gluconolactone hydrolysis in the absence (red) and presence (blue) of 0.6 mg GLA at 25 °C. (B) Change of $[H^+]$ during the course of the reaction. (C) δ-gluconolactone hydrolysis rate (first derivative of $[H^+]$ with respect to the time) dependence on pH. The enzyme was isolated from baker's yeast and purified according to [1]. The temperature of the reaction mixture was controlled by a Peltier element. 5 mM glucose was used as a substrate for the pairing enzyme, glucose oxidase (0.1 µM), that produced δ-gluconolactone. The pH values were measured by a pH meter HI 9017 connected to a 5-mm in diameter combination pH electrode (HI 1330B). Data were collected using a HI 92000 software.

In our experiment, various protein fractions were analyzed for the presence of gluconolactonase during its isolation from the yeast (Figure 2A). The kinetic results have been compared with the results from SDS-PAGE (Figure 2B). In the gel, the protein bands were

analyzed by ImageJ software and the 165-kDa band (expected MW of gluconolactonase) density has been correlated with the reaction rate data. The results show that only protein fractions containing gluconolactonase exhibit reaction rates exceeding the control value without the enzyme.

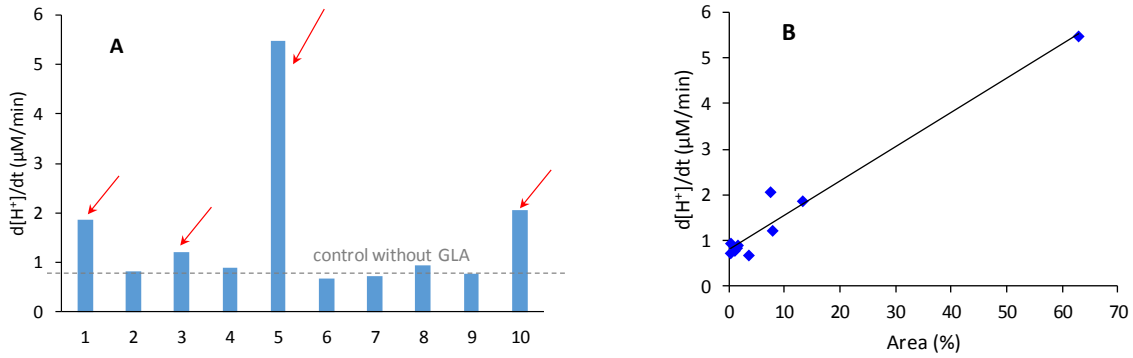


Fig. 2. (A) Reaction rates of gluconic acid dissociation observed at pH 5.2 (25 °C) in ten protein fractions during GLA isolation. The arrows point to the fractions containing GLA. Dashed line is a value corresponding to the enzyme-free reaction rate (the rate of a spontaneous hydrolysis of gluconic acid). The amount of proteins in each sample was 0.6 mg. (B) Correlation of the reaction rate with the 165-kDa band area from SDS PAGE.

The main advantages of the method are simplicity of the experiment, sensitivity of the technique and possibility to obtain data over a wide pH range in one experiment. We have shown that the potentiometry is an effective technique in the study of enzyme kinetics and that it might be used in any enzyme-catalyzed reaction related to pH changes.

Acknowledgement
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The effect of insecticide thiacloprid on the stability of DNA – preliminary study

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Currently, the pesticides are routinely used to protect agricultural crops from pests. Their disadvantage is that they pose a potential risk to humans and animals due to the presence of their residues in the food [1]. We have focused on the study of the effect of thiacloprid (TCL), which is the active ingredient of the pesticide preparation Calypso 480SC, on the structure and stability of important biomacromolecules as the genetic material represented by nucleic acids.

Thiacloprid (3-(6-chloro-3-pyridinylmethy)-2-thiazolodinylidene)cyanamide) belongs to a group of neonicotinoid insecticides, especially to the group of chloronicotinyl insecticides. It accounts wide-spectral effects. Thiacloprid is a preparation for the protection of rapeseed oil, fruit trees, vines, vegetables, potatoes and ornamental woods against mammalian and insect pests, especially toads and flies [1]. TCL is systemic insecticide that enters the body through intimate contact or ingestion. It acts as a nicotine acetylcholine receptor agonist (nAChR). Its mechanism of action is based on disruption of controlled synaptic transmission by irreversible binding to the nACh receptor and attacks the nervous system of insects [2]. Neonicotinoids have low affinity for nAChR vertebrates at physiological pH, because they are not protonated. As a result, they show low acute and chronic toxicity in mammals, birds and fish. Nevertheless, it has been shown that thiacloprid exhibits a relatively high level of acute toxicity for fish [3]. After ingestion, TCL is rapidly absorbed, widely distributed inside the body, extensively metabolized and secreted in the urine. The target organ is liver [4]. Significant DNA damage was observed for *Eisenia fetida*, we can predict that thiacloprid may have a harmful effect on earthworms [5]. Kocaman et al. (2012) reported that thiacloprid showed a cytotoxic/cytostatic effect in human peripheral blood lymphocytes probably due to the increased levels of DNA damage, and that this effect resulted probably from the inhibition of DNA synthesis and cell proliferation. Because the use of thiacloprid has become increasingly widespread throughout the world, the assessment of its possible genotoxic and cytotoxic effects on living organisms is very important [6].

In this work, we have studied the effects of thiacloprid on the DNA. This interaction was studied by using fluorescence spectroscopy and absorption spectroscopy with Peltier module. Fluorescence spectra indicate the decreases of DNA emission with the increasing thiacloprid concentration. To determine the strength and mode of interaction we constructed the Langmuir isotherm and calculated the value of the binding constant according to Copeland (2000) [7]. Based on both graphical approaches, the binding constant of the DNA/TCL complex was determined as $4.63 \cdot 10^4$ l/mol. In comparison with typical DNA intercalators such as actinomycin D (binding constant $K_b = 1.9 \cdot 10^6$ l/mol) [8] or mitoxantron ($K_b = 3.9 \cdot 10^5$ l/mol) [9] our obtained values do not point to intercalation mode of the DNA/TCL interaction. Drugs binding by weak electrostatic forces to the surface of DNA exhibit lower binding constant (naringin $K_b = 3.1 \cdot 10^3$ l/mol [10]; vincristine $K_b = 1 \cdot 10^3$ l/mol [11]). In view of the above, we

can suppose that thiacloprid binds to the DNA groove, since the value of the binding constant corresponds to this type of binding [12].

In addition, we proved our results by denaturation of pure DNA and DNA/TCL complexes in ratios 1/1, 1/2, 1/3 and 1/5 by absorption spectroscopy connected with a Peltier module. Melting curves (not shown) illustrate destabilizing effect of the pesticide on calf thymus DNA. They were fitting using Van´t Hoff equation to obtain main thermodynamic characteristics (T_m, T, H)

$$A = A_{min} + \frac{A_{max} - A_{min}}{1 + e^{\left[\frac{\Delta H}{R} x \left(\frac{1}{T} - \frac{1}{T_m}\right)\right]}}$$

where A - absorbance, A_{min} - minimal measured absorbance, A_{max} - maximal measured absorbance, H - enthalpy of transition, R - gas constant, T - temperature, T_m - melting temperature. Melting temperature T_m is shifted from 64.5 °C (pure DNA) to 61.9 °C for the complex DNA/TCL in 1/2 concentration ratio. Also Van´t Hoff enthalpy H is decreasing from 413 kJ (pure DNA) to 390 kJ (DNA/TCL), which means that lower energy is needed for denaturation of 50 % base pairs of DNA in the complex with thiacloprid. The changes in thermodynamic parameters lead us to a claim that thiacloprid destabilizes the calf thymus DNA and this destabilization is associated with the groove binding interaction mode [13]. In our experiments, the melting curve of the complex DNA/TCL in 1/3 and 1/5 shows a two-phase character with an expressive destabilization of AT regions in DNA. It is generally supposed that small molecules like pesticide thiacloprid bind preferentially into the minor groove of DNA, especially to regions that are rich in AT base pairs [14].

The neonicotinoid insecticide thiacloprid influence on structure and denaturation stability of the DNA macromolecule points to the groove binding mode of interaction and the incorporation of the drug takes place probably into the DNA minor groove by hydrophobic or hydrogen interactions. Our findings can be supported by the fact that an intercalative mode of interaction manifests in higher value of binding constant and thermal stabilization of the DNA molecule due to ligand binding [13]. These conclusions are the results of preliminary study. This problem requires futher investigation.

Acknowledgement
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Model membrane perturbation studied by fluorescence spectroscopy

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The first phase of solubilization process (detergent partitioning and membrane perturbation) was observed using fluorescence spectroscopy. Unilamellar liposomes (ULL), consisting of dioleoylphosphatidylcholine (DOPC) with/without cholesterol (CHOL), were used for mimicking the lipid part of the mammalian cytoplasmic membrane. Membrane perturbation was caused by adding antimicrobially active detergent *N*,*N*-dimethyldodecanamine *N*-oxide (DDAO) to ULL dispersion at pH 7.5. Fluorescent probe leakage method [1] was used to observe perturbation of model membranes. DOPC and DOPC+CHOL (CHOL : DOPC = 0,5 mol/mol) ULLs were filled with the fluorescent probe, calcein, at a self-quenching concentration. Unencapsulated probe was removed by gel chromatography. For each type of model membrane, we prepared 4 sets of samples with varying lipid concentrations. Each set consisted of samples with constant lipid concentration and increasing amount of DDAO. DDAO partitioned between the water and lipid environment and with the increasing amounts of DDAO incorporated into the bilayer, it induced the formation of defects and pores in the bilayer. Fluorescent probe was leaking though the pores into extraliposomal area. Diluting the probe in the extraliposomal area enabled the increase in the fluorescence intensity to be observed. Two turning points were observed on the dependence of the fluorescent intensity on the concentration of added DDAO. The first turning point, D_T^{PERT}, determines the concentration of DDAO causing calcein to wash out heavily from ULLs. The second turning point, D_T^{RLS}, determines the concentration of DDAO that causes the equalization of extraliposomal and intraliposomal concentration of calcein. The fluorescence intensity does not increase with further DDAO addition. Using these turning points, partition coefficients of DDAO were calculated for both types of membranes. [2] Effective ratio of the DDAO amount integrated into the bilayer to the amount of lipid were determined. The results suggest that replacing of 1/3 of the DOPC molecules by CHOL molecules does not significantly affect the partition coefficient.

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Study of fungicide tebuconazole effect on human serum albumin

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Pesticides have long been used in agriculture to increase quality and quantity of crops. It is known, that even low doses of pesticides activate biochemical changes. These changes can be responsible for many health effects to humans and animals. Knowledge about mode of action and consequences of their using is necessary to avoid pesticides application in inappropriate places [1].

In this work, we have studied the effect of selected fungicide tebuconazole (TB) on the human serum albumin (fatty acid free). The interaction was studied by spectroscopic methods thathave become very popular methods for revealing protein-ligand interactions because of their high sensitivity, reproducibility, rapidity and convenience.

Systemic pesticide tebuconazole [1-(4- chlorophenyl)-4,4- dimethyl-3- (1,2,4-triazol- 1-ylmethyl)pentan- 3-ol] is a representative of triazoles [2]. This class of fungicides is widely used in crops protection. Due its broad-spectrum antifungal activity itis applied on number of crops such as vegetables, fruits and rice. Tebuconazole inhibits the enzyme lanosterol-14-α-demethylase which plays an important role in sterol production in cells. As a consequence of these processes an inhibition of fungi growth is reached [3].

Human serum albumin (HSA) is the most abundant plasma protein and contributes to significantly many transport and regulatory processes. The protein binds a wide variety of substrates such as metals, fatty acids, amino acids, hormones and an impressive spectrum of drugs [4].

After exposure of human body, triazole fungicides may penetrate into the blood stream and interact with plasma proteins, they could have an impact on the structure and function of proteins [5]. The investigation of TB binding with proteins has toxicological importance. This study is expected to provide an important insight into the interactions of the transport protein HSA with fungicide.

Our experimental results show the fluorescence quenching of HSA by TB. The binding parameters and thermodynamic parameters of a complex HSA/TB formation were obtained from fluorescence measurements at three temperatures (25°C, 30°C and 37°C). The temperature-dependent fluorescence spectra manifested static type of quenching, which is a dominant mechanism for fluorescence quenching and indicates the complex formation (Fig.1). On the other hand a dynamic type of quenching is caused by the changes in environment of binding site.

The binding parameters were determined by Hill plot characterizing by equation 1

$$log\left(\frac{F_0-F}{F}\right) = logK_A + nlog[Q] \qquad (1)$$

where K_A represents the association constant, n is the number of binding sites, $F_0$and F are the fluorescence intensities of protein in the absence and presence of quencher, $[Q]$ is the concentration of quencher. Association constants of HSA/TB at different temperatures are valuated from 0.851 to 0.346 x 10^4mol/l. These values represent a slight binding force between TB molecule and macromolecule of HSA.

With concern to mode of interaction between both molecules thermodynamic parameters were calculated using equations

$$\ln K_A = \frac{-\Delta H}{RT} + \frac{\Delta S}{R} \quad (2)$$

$$\Delta G = \Delta H - T\Delta S \quad (3)$$

where ΔH is enthalpy change, ΔS is entropy change, ΔG if free energy change, T is temperature and R is the universal gas constant [5]. Negative values of enthalpy as well as entropy changes point at hydrogen bonding of tebuconazole into HSA [5].

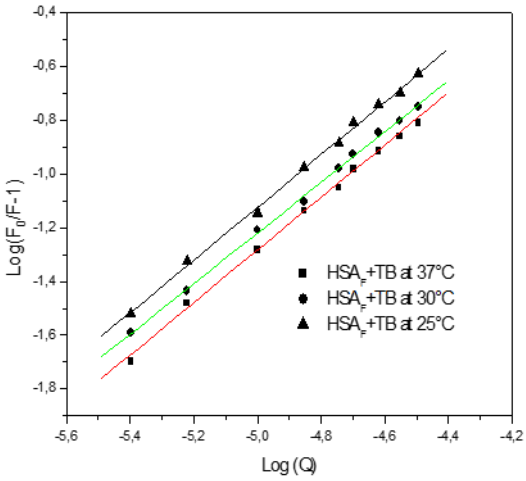

Fig.1 Hill plots for determination of binding parameters of HSA interaction with TB

The results of synchronous fluorescence spectroscopy show that TB binds in the vicinity of Trp residues. Furthermore, the three-dimensional fluorescence spectra indicate (not shown) slight changes in secondary structure of HSA.

Our results indicate the slight binding affinity between TB and HSA, but they are in good correspondence with binding parameters of other triazole fungicides [5].

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