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BIOCATALYSIS: FUNDAMENTALS & APPLICATIONS

12th INTERNATIONAL CONFERENCE

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ABSTRACTS

June, 24-28, 2019
St. Petersburg - Valaam - Kizhi
Russian Federation

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LECTURES ABSTRACTS

Novel approaches for screening biodiversity

Alexander Gabibov

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Combinatorial chemistry and biology became a hallmark of life science in XXI century. To screen large repertoires of proteins and cell clones we need to design sensitive detection systems and screening platforms. We developed microfluidic approaches for screening microbiota, biocatalytic clones, antibody diversity and specific chimeric antigen receptors (CARs). We report the development of a novel platform to significantly enhance the efficacy and safety of Follicular lymphoma treatment. Since lymphoma is a clonal malignancy of a diversity system every tumor has a different antibody on its cell surface. Combinatorial autocrine-based selection is used to rapidly identify specific ligands for these B cell receptors on the surface of FL tumor cells. The selected ligands are used in a CAR-T format for redirection of human CTLs. Essentially, the format is the inverse of the usual CAR-T protocol. Ultrahigh-throughput screening (uHTS) techniques can identify unique functionality from millions of variants. To mimic the natural selection mechanisms that occur by compartmentalization in vivo, we developed a technique based on single-cell encapsulation in droplets of a monodisperse microfluidic double water-in-oil-in-water emulsion (MDE). Biocompatible MDE enables in-droplet cultivation of different living species. The combination of droplet-generating machinery with FACS followed by next-generation sequencing and liquid chromatography-mass spectrometry analysis of the secretomes of encapsulated organisms yielded detailed genotype/phenotype descriptions. This platform was probed with uHTS for biocatalysts anchored to yeast with enrichment close to the theoretically calculated limit and cell-to-cell interactions. The versatility of the platform allowed the identification of bacteria, including slow-growing oral microbiota species that suppress the growth of a common pathogen, Staphylococcus aureus, and predicted which genera were associated with inhibitory activity. Next, we developed a novel platform for maturation of antibody molecule in silica. In vitro selection of antibodies from large repertoires of immunoglobulin (Ig) combining sites using combinatorial libraries is a powerful tool, with great potential for generating in vivo scavengers for toxins. However, addition of a maturation function is necessary to enable these selected antibodies to more closely mimic the full mammalian immune response. We approached this goal using quantum mechanics/molecular mechanics (QM/MM) calculations to achieve maturation in silico.

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Enzymes, radicals, deseases and agening

Vladimirov Yuri

New thermophilic prokaryotes, new metabolic pathways

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Natural thermal environments harbor unique communities of prokaryotes, with extremely diverse metabolic abilities. Of especial interest are microorganisms utilizing energy substrates and electron acceptors of volcanic origin.

Numerous new isolates inhabiting terrestrial and submarine hot environments are lithoautotrophic anaerobes growing by the reduction of sulfur compounds with molecular hydrogen. Of special interest is *Thermodesulfitimonas autotrophica* – the first bacterium, obligately dependent on sulfite as the electron acceptor.

Several new microorganisms representing new genera in diverse phylogenetic groups and capable of growth by sulfur compounds disproportionation were isolated and characterized. They can grow using elemental sulfur, thiosulfate and sulfite both like electron donors and acceptors; the products of these reactions are hydrogen sulfide and sulfate.

Lithoautotorphic thermoacidophilic sulfate-reducing bacteria representing new genera were isolated from Kamchatka hot springs and found to be able to grow at pH 3.0 reducing sulfate with molecular hydrogen as the growth substrate. One of these microorganisms, *Thermodesulfobium acidiphilum,* performs a new version of Calvin cycle, where the key enzyme, ribulose-bis-phosphate carboxylase (RubisCO), is represented by the Form III, previously known only as an enzyme participating in nucleotides metabolism in archaea.

Utilization of xanthan-gum as a substrate allowed us to isolate several new genera of thermophilic planctomycetes, among them a representative of a new genus, *Thermogutta terrifontis*. Transcriptomic analysis of *T. terrifontis* growth on xanthan gum allowed us to propose a new pathway of this compound hydrolysis.

Extremophilic enzymes for resource-efficient, robust and selective biocatalytic synthesis of small molecules

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Keywords: Hydrolase, Dehydratase, Kinase, Metabolites, Systems Biocatalysis

The universe of enzymes from extreme environments is of much interest for discovering robust and highly stable enzymes which can be applied in resource-efficient syntheses of small molecules and are able to work efficiently, also under non-conventional reaction conditions. Metabolic pathways in microorganisms from extreme environments are an excellent blueprint from nature for the synthesis of metabolites and metabolite-like small molecules. Starting from a systems biocatalysis approach, key success factors for the design of viable new biocatalytic routes for the synthesis of small molecules are a comprehensive retrosynthetic analysis and the selection of suitable enzymes. Although this selection is supported by the rapidly growing databases, it is essential to have also highly active enzymes available, to have experimental evidence of their functions and to interface their functions and properties to the design of biocatalytic processes under reaction conditions.

A selection of hydrolases, dehydratases and kinases, the genes of which originated from a diversity of microorganisms in hot environments, have been expressed in *Escherichia coli*, thus allowing easy purification towards highly active enzymes. Process analytical tools with high information content, such as LC-MS and NMR, have been instrumental for rapid prototyping of new preparative biocatalytic reactions. Reaction engineering and process intensification have been useful in the further development towards highly selective and straightforward synthetic methodologies for biocatalytic hydrolyses, water eliminations and phosphorylations. This has been demonstrated by numerous applications, such as the synthesis of all four limonene oxide enantiomers and vicinal limonene diols using recombinant epoxide hydrolases with complementary stereoselectivity in biocatalytic ring-opening reactions, superior to chemical routes. The biocatalytic one-step synthesis of chiral 2-keto-3-deoxy-sugar acids from easily available carbohydrate precursors by water elimination provides a great advantage compared to the multitude of reaction steps required in their stereospecific chemical synthesis, such as the synthesis of 2-keto-3-deoxy-D-gluconate (KDG) from D-gluconate using recombinant gluconate dehydratase from *Thermoproteus tenax*. In the area of biocatalytic asymmetric phosphorylations, microbial kinases have enabled straightforward and protecting-group free phosphorylations in fewer reactions steps and with higher purity than chemical routes, such as the synthesis of D-glycerate-2-phosphate in a highly enantioselective phosphorylation of glyceric acid catalyzed by glycerate 2-kinase form *Thermotoga maritima*, which was expressed in *E. coli*.

The power of extremophilic enzymes provides not only great new tools for improving resource efficiency in the science of synthesis of small molecules, but offers also treasure islands for finding known enzyme functions with novel enzyme characteristics applicable to predefined reaction conditions as well as for discovering completely novel enzyme functions which have not yet been classified by the Enzyme Commission.

Neuroenzymology: kinetics of biocatalytical reactions in human brain

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Competing substrate kinetics for michaelian enzymes and for enzymes showing activation/inhibition by excess substrate: application to kinetic analysis of cholinesterases

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Kinetic analysis of enzyme catalytic behavior from integrated rates of progress curves has been little used until it is possible to integrate mathematically or numerically the rate equations. This approach was even less used in the case of reactions involving competition between two substrates. We have developed a formalism which makes possible to investigate the effect of a 'dark' ('invisible') competitor substrate (B) on the progress curves of a reporter ('visible') substrate (A). This approach is particularly suitable when there is no simple spectrophotometric method to analyze the kinetics of degradation of 'invisible' substrates, as well as for study of poorly water-soluble or highly toxic substrates.

Rate equations were integrated for Michaelis-Menten kinetics and for enzyme activation or inhibition by excess of B (Webb-Radic equation, involving substrate binding on a secondary site that allosterically affects k_{cat} as $b.k_{cat}$ with b>1 for activation and b<1 for inhibition). The shape of progress curves for degradation of A in the presence of B depends on the ratio of specificity constants $(k_{cat}/K_m)_{B/A}$, the competition matrix (R). Mathematical solutions exist for $R>>1$, $R = 1$, $R<<1$.

Working at constant substrate A concentration, from the shape of progress curves (sigmoidal or non-sigmoidal), it is possible to define the type of competitor (B), and from the dependence of retardation time of progress curves (at 90% completion of A, and at inflexion point for sigmoid-like shaped progress curves) on substrate B concentration, it is possible to access to all catalytic parameters, and/or to titrate enzyme active sites. Moreover, $K_{m,A}$ or $K_{m,B}$ can be determined from initial velocity ratio (v_i/v_0 *vs* [B]).

Competiting substrate kinetic analysis of human cholinesterases (AChE and BChE), using classical UV-visible spectrophotometric methods is described. The approach is based on the use of a chromogenic substrate A whose complete hydrolysis time course is retarded by a competing substrate B. Different 'visible' and 'invisible' substrates were used, including thio/oxo-esters and arylacylamidates. The ratio of bimolecular rate constants (R) for the different couples of substrate (B/A) covered all cases of limit situations, $R<<1$, $R\approx1$ and $R>>1$. Binding and catalytic parameters of enzymes for either michaelian (b=1) or non-michaelian behaviors (b>1 or b<1) by excess [B]) were easily determined. Moreover, new mechanistic information on the significance of the activation/inhibition 'b' factor were obtained.

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New Biocatalysts for Processes of Fine Organic and Chiral Synthesis with Oxidoreductases

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Enzymes are highly efficient biocatalysts but different processes require enzymes with different properties and native sources can not provide them. Moreover, high cost of enzymes preparation (especially for chiral synthesis and pharmaucetical industry) very often prevent biocatalysts introduction into practice. Protein engineering methods permit to get biocatalysts with prescribed properties but initial source plays important role because volume (and cost) of work depend on properties of initial enzymes. In our our laboratory we carry out systematic works to find new sources of enzymes with the most suitable ofr target process. Bioinformatic search and selection of genes of interest are followed by cloning, overexpression. Gene structure is modified to improve expression level, stability and activity of enzyme. Crystallization and determination of 3D structure provide background for rational desigh of enzymes. Using this approach we found and cloned genes of new formate dehydrogenases and D-amino acid oxidases with new properties. Five crystal structures were determined.

The second direction of experiments is creation of new hybrid biocatalysts which are not observed in nature. We prepared hybrid chiral biocatalysts consisting on oxidoreductase (two types of monooxigenases) with two types of mutant $NADP^+$-dependent formate dehydrogenases. Genetic construction had different order of genes. Hybrid enzymes were expressed and purified as active soluble proteins. In one case lengh of polypeptide chain was about 1200 amino acid residues. New hybrid biocatalysts showed higher catalytic efficiency compared to separate enzymes.

This work was supported by Russian Foundation for Basic Research (grants 17-04-01469a, 17-04-01487a, 17-04-01662a, 18-34-00594 and 18-34-20098_mol_ved), RFBR and Moscow city Government (grant 19-34-70036), Russian Science Foundation (grant 18-74-00146) and DAAD (fellowships for PDP).

Knowledge-based modulation of enzyme functional properties

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Keywords: enzyme functional properties, modulation

Modulation of enzyme functional properties is a challenging problem for both fundamental enzymology and enzyme engineering. The task can be solved by changing protein structure at mutation or modification, due to interaction of an enzyme with modulating molecules or a carrier, etc. To settle these problems we propose to use interdisciplinary approach that combines the strength of bioinformatics, molecular modeling, high performance computing, theoretical chemistry, protein engineering and is currently implemented as a platform of 8 public web-servers [1-8]. The developed methodology was applied for the different purposes [9-11]: i) to study structure-function relationship in several enzyme families (Ntn-hydrolases, penicillin-binding proteins, sialidases, α/β-hydrolases), ii) to search for function-related variable positions in protein structure that can be used as hotspots for mutations to modulate catalytic activity, substrate profile, stereoselectivity and stability of enzymes, iii) to identify previously unknown binding sites in enzyme structure for binding of modulating ligands that can be used to design selective inhibitors. The report will summarize laboratory's experience in building up and application of such a platform to create biocatalysts with improved properties and to design new selective inhibitors of enzymes of pathogens. The developed methodology can be recommended as a systematic tool to study structure-function relationship, characterize and rank enzyme binding sites for binding of new selective modulating molecules, identify function-related positions and use them as hotspots for mutation to rationalize different protein engineering approaches thus designing enzymes with requested functional properties.

This work was supported by the Russian Science Foundation (grant #15-14-00069-P)

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Beta-lactamases - drivers of bacterial antibiotic resistance

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Keywords: beta-lactamases, antibiotics, antibiotic resistance, mutations, allosteric inhibitors

Bacterial resistance towards antibiotics has become a global problem in both the medical and agricultural fields, and resistant bacterial strains present an increasing threat to humans and animals. β-Lactam antibiotics (penicillins, cephalosporins, carbapenems, monobactams), being the most commonly used, irreversibly inhibit penicillin-binding proteins (PBPs) participating in the synthesis of the cell wall, and this leads to the death of bacteria. The bacterial resistance to beta-lactams develops due to the production of the superfamily of bacterial enzymes – beta-lactamases (BLs). These enzymes are evolving at high rate and have noticeable structural variability. To date, more than 2,700 BLs, differing in structure, catalytic activity and resistance to inhibitors, have been described. They may be considered as drivers of the resistance due to the high mutation rate of existing serine BLs, appearance of new types of enzymes with wider subsrate specificity (metallo-BLs), and fast spread of resistance due to horizontal gene transfer. All these factors explain exponentional increase of resistance among pathogenic bacteria and represent currently a global threat. The variability of BLs is a consequence of general biological mechanism of adaptation of microorganisms to antibiotics. Due to the increasing diversity of BLs, a more in-depth study of the mechanisms of antibiotic resistance is being carried out using methods of mega-science, in particular XFEL.

BLs and PBPs had a common predecessor, and their evolutionary relationship consists in maintaining a similar conservative structural fold of secondary structure elements connected by flexible loops. Their common property is the ability to acylate catalytic serine during the interaction with an antibiotic, and the main difference of BLs consists in their ability to deacylate the acyl-enzyme complex. In the course of evolution, BLs changed the active site: some structural elements disappeared and new ones appeared, in particular, the omega-loop at the entrance to the active site. This loop in class A BLs contains a conservative charged amino acid residue E166, which plays a key role in the deacylation of the acyl-enzyme complex. This loop is also present in other serine BLs belonging to classes C and D.

A family of TEM-type beta-lactamases involves currently more than 220 enzymes, which are mutant forms of beta-lactamase TEM-1. We have created a biobank of recombinant forms of TEM type BLs, including single substitutions and their combinations. The use of structural analysis, molecular dynamics, and methods of enzymatic kinetics made it possible to study the effect of combinations of the key and secondary mutations on the catalytic activity and stability of the enzymes. Particular attention was paid to the analysis of mobility of omega-loop – important structural element located at the entrance to the active site. its conservative residue E166 participates in the acylation/ deacylation processes forming the network of hydrogen bonds for the activation of catalytically important residues and hydrolytic water molecules. Despite differences in the primary sequence, the conformational folding of omega-loops is the same for different class A BLs. Targeting the omega-loop has the potential for allosteric inhibition of the BLs with a new class of effective antimicrobials.

The expression of BL genes was also studied for laboratory strains-producers of TEM type BLs and clinical strains producing several BLs simultaneously. Inhibition of BL expression factors, including effects on regulatory histidine kinases, is a new approach to overcome the bacterial resistance.

The work was supported by the RSF (Project No. 15-14-00014-P).

Development of new enzyme mechanisms of animal antiviral resistance using African swine fever virus model

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Keywords: antiviral resistance, African swine fever virus (ASFV), protease S273R, procaspase-3, single molecules measurement

Human and animal emerging infectious diseases pathogenesis study and development of specific treatments rely on multidisciplinary approaches. The ambitious goal was set up to develop novel molecular mechanisms of animals antiviral resistance to African swine fever virus (ASFV). To achieve this goal, the research was focused on studying procaspase-3 mutant forms having recognition site for ASFV for processing and activation by ASFV protease S273R in vitro in mammalian cells by the fluidic system for single molecules measurement. We have generated ASFV S273R protease producer, various procaspase-3 forms, and also swine inhibitor apoptosis proteins BIR2 and A224L of ASFV. Variants of procaspase-3 were selected based on the efficiency of processing by ASFV S273R protease. The protease S273R is expressed in monkey cell line COS-1, however without visible detection of its activity. The protein expression analysis has demonstrated that modified procaspase-3 is a target of endogenous processing and activation. At the same time, catalytic mutant and native processing site mutant have been expressing as proenzymes and haven't been activated by endogenous mechanisms. As long as virus protease S273R was inactive without viral infection in mammalian cell line, we could not analyze an activation of modified procaspase and apoptosis induction in vitro. We studied processing and activation modified procaspase-3 in cell lysates with protease S273R, extracted from E.coli. The results have shown, that the protease S273R split modified procaspase-3 using interdomain linker, that leads to caspase-3 activation in the cell lysates. For research of the enzymatic properties of created proteins using single molecule detection based on TIRFM method, we generate caspase-3 enzyme with biotin tag on the C-terminus of one of the chains. In compliance with that, we have designed the expression system to produce procaspase-3 in a heterodimeric form: one subunit is procaspase-3 fused with biotinylated peptide on the C-terminus, whereas another subunit tagged with eGFP molecule and calmodulin binding peptide on the C-terminus. For more efficient procaspase-3 heterodimer biotinylation in vitro we constructed the expression plasmid with birA gene of E.coli biotin-ligase. We developed the single molecule detection system to determine catalytic constants of the caspase-3 and its mutant forms in single molecule mode. The microfluidic system was designed to detect immobilized enzymes. We obtained active conjugates of porcine caspase-3 with fluorescent dyes (Cyanine3, Cyanine5) and biotin. The immobilization conditions of caspase-3 conjugates and their detection on cover glass were also optimized. The average lifetime of the fluorescent molecule on the substrate was 20 s with 4 mW of excited light power after the lens. This time was sufficient for enzyme activity detection in a particular point.

This work was supported by RFBR grant № 17-29-08010.

Diversity of mechanisms in enzyme-catalyzed phosphorus-oxygen bond cleavage reactions

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Keywords: enzyme catalysis, reaction mechanism, P-O bond cleavage, molecular simulations

We consider several enzyme-catalyzed reactions which include the step of phosphorus-oxygen bond cleavage either in substrate molecules or in the protein side chains. Reaction mechanisms are discussed by results of experimental studies and computer molecular simulations based on quantum mechanics/molecular mechanics and molecular dynamics approaches. All-atom molecular models for simulations are constructed by motifs of relevant crystal structures. Energy profiles computed along the corresponding reaction coordinates allow us to formulate mechanisms of chemical transformations in enzyme active sites.

Reactivation reactions of phosphorylated cholinesterases constitute an important class of the processes with the P-O bond cleavage. Wild-type and mutated proteins are studied in attempts to suggest novel efficient bioscavengers.

Hydrolysis reactions of nucleoside phosphates include multiple examples. One of the most important processes is the hydrolysis of guanosine triphosphate (GTP) by small GTPases. In particular, the mechanism of GTP hydrolysis by the Ras-GAP complex is studied in details.

Conversion of ATP to cAMP by adenylyl cyclases employs one more type of reactions with the phosphorus-oxygen bond breaking.

We demonstrate that these reactions are characterized by a remarkable diversity of molecular mechanisms.

This work is supported by the Russian Science Foundation (project 19-73-20032).

The emitter as an intramolecular probe in the luciferase active center

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Keywords: bioluminescence, firefly luciferase, keto-enol tautomerization, oxyluciferin.

Investigation of the firefly bioluminescence spectra and the emitter structure is one of the important problems already in the first researches on the reaction mechanism. The studies of natural and model bioluminescent systems showed that bioluminescence of firefly luciferases is characterized by complex changes of the emission spectra and bioluminescence λ_{max} occurring with changing pH, temperature, and the enzyme structure. The firefly bioluminescence maximum in nature varied in a wide range. So, the conclusion was made that the bioluminescence spectra were defined by microenvironment of the emitter in the enzyme active center. Already at the start of 21st century substantial experimental evidence has been accumulated corroborating contribution of the keto-enol tautomerization mechanism to the firefly bioluminescence. The numerous experimental results suggested that the red emission is related to the keto-form, while the yellow-green emission – to the enol/enolate form of the emitter. Despite the large number of publications considering the structure of bioluminescence emitter based on keto-enol tautomerization, several authors suggested alternative hypothesis on the emitter structure: 1) the keto-form of oxyluciferin is the only emitter in this system, which could exist as two stereo-conformations with the planar (green emitter) and perpendicular (red emitter) mutual arrangement of the benzothiazole and thiazole rings; 2) the different resonance structures of the oxyluciferin keto-anion generate green and red bioluminescence; 3) the entire variety of the firefly bioluminescence spectra theoretically is explained by polarization of the OH-bond in the protein microenvironment in any form of the emitter; 4) the spectrum of enolate ion can cover the entire range of yellow-green and red bioluminescence. However, novel experimental evidence for existence of keto-enol tautomerzation of oxyluciferin in solution and in complex with luciferase appeared in the literature and the results of our own research allowed concluding that keto-enol tautomerization of the excited oxyluciferin molecule provides the most adequate explanation of the observed complex spectral changes.

Only one emitter molecule may be present in the active center of the luciferase molecule, hence, the emitter can be considered as an intramolecular probe reporting on the properties of its microenvironment in the enzyme active center. Superposition of two or three forms of the emitter recorded in the bioluminescence spectra implies that equilibrium of different conformations of the enzyme exists in the reaction medium:

$$\mathbf{E_1 \leftrightarrow E_2 \leftrightarrow E_3}.$$

Each luciferase conformer contains one molecule of electronically exited oxyluciferin:

$$\mathbf{E_1(LO{=}O)^* \leftrightarrow E_2(LO\text{-}OH)^* \leftrightarrow E_3(LO\text{-}O^-)^*.}$$

The relative content of each form of the enzyme-emitter complex can varied with the pH change in accordance with the shift of equilibrium between the three forms of luciferase molecule.

Site-directed or random mutagenesis of the luciferase globule may change the ratio between the different enzyme conformers and shift the spectrum towards green or red bioluminescence. For example, the single Tyr35His mutation resulted in formation of the mutant *L. mingrelica* luciferase with monomodal spectrum in the entire pH range 6.0–7.8. It shows that this mutant exists as a single conformer, while the initial luciferase molecule contains two different conformers depending on the pH. Another example: the comparison of the bioluminescence spectra for luciferase single-point mutants at different temperatures showed that $\lambda_{max.em}$ for the green and red components were the same as for initial luciferase, and only the ratio between these components was changing with the increase of temperature. It indicates that the emitter structure does not depend on the temperature, but the ratio between the different luciferase conformers is temperature-dependent.

Nanotechnology Platforms in Cancer Therapy (Engineering Targeted Polymeric Nanocarriers for Drug Delivery)

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Nanoscale polymeric particles, in particular self-assembled block copolymer micelles, have been utilized in pharmaceutics for development of novel therapeutic and diagnostic modalities. Advantages of the polymeric micelles include their small size, long circulation in bloodstream, ability to circumvent renal excretion and extravasation at sites of enhanced vascular permeability. They can be designed to facilitate the incorporation of a variety of compounds or even particles through a combination of electrostatic, hydrophobic, and hydrogen bonding interactions. Micelle structure can be tuned to control the drug-release characteristics of the micellar carriers. Chemical functionalization of polymeric micelles with targeting moieties was also explored in order to enhance accumulation of highly toxic ingredients specifically within cancer cells and prevent their accumulation in healthy organs. The potential application of such polymeric micelles as carriers for multidrug delivery will be discussed.

Smart Nanomaterials for Biomedical Application

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Keywords: ribonuclease, nanoparticles, radionuclide, toxins, theranostics

Targeted therapy makes use of high-affinity molecules as carriers of therapeutic agents to tumor cells. In frame of this approach we designed a family of fully genetically encoded targeted imaging and therapeutic compounds based on anti-HER2 tumor-specific polypeptides (single-chain variable Fragment - scFv, designed ankyrin repeat protein - DARPin, Affibody) and toxic principles: protein photosensitizers (Killer Red, miniSOG), a ribonuclease barnase, exotoxin A from Pseudomonas aeruginosa. Supplementary, a novel strategy, "Protein-assisted NanoAssembler", for design of heterostructures based on the ribonuclease barnase and its inhibitor, barstar, was suggested. The barnase and barstar are small, stable, very soluble, resistant to proteases proteins. The complex between them is extremely tight with a Kd~10-14 M. The N- and C-terminal parts of both proteins are localized outside of the barnase·barstar interface and are therefore accessible for fusion with targeting, visualizing or toxic compounds. The suggested strategy is applicable to virtually any proteins that can be functionally attached to the barstar and barnase molecules. It seems particularly well suited to the production of heterooligomeric constructs because the extremely specific barnase·barstar interaction eliminates reliably the mispairing problems.

A particular attention as new and unique therapeutic agents attract nanoparticles (NPs) that make it possible to solve old but still actual problems by principally new means and ways. In the presentation we review our recent results on applications of multifunctional agents with important types of the nanoparticles, including quantum dots (QDs), luminescent nanodiamonds (LNDs), colloidal gold, magnetic NPs, and luminescent upconversion NPs (UCNP). An emerging new-generation approach in biomedicine, called theranostics, relies on the detection of a complex consisting of a carrier molecule and toxic agent. The theranostics concept is realized by employing drugs with dual therapeutics and diagnostics functionality. Despite significant side effects, traditional chemo- and radiotherapy remain most widely used therapeutic modalities of cancer therapy owing to their high efficacy. The use of two toxic drugs is known to result in a superadditive effect, with a total effect of the drugs (Rab) greater than an arithmetic sum of the constituent toxic effects (Ra, Rb), so that Rab > Ra + Rb.

We report combined therapy using upconversion nanoparticle (UCNP) coupled to two therapeutic agents: beta-emitting radionuclide yttrium-90 (90Y) fractionally substituting yttrium in UCNP, and a fragment of the exotoxin A derived from *Pseudomonas aeruginosa* genetically fused with a targeting designed ankyrin repeat protein (DARPin) specific to HER2/neu receptors. The resultant hybrid complex UCNP-R-T was tested using human breast adenocarcinoma cells SK-BR-3 overexpressing HER2/neu receptors and immunodeficient mice, bearing HER2-positive xenograft tumors. The photophysical properties of UCNPs enabled background free imaging of the UCNP-R-T distribution in cells and animals. Specific binding and uptake of UCNP complexes in SK-BR-3 cells was observed, with separate 90Y- and PE40-induced cytotoxic effects characterized by IC50 140 μg/mL (UCNP-R) and 5.2 μg/mL (UCNP-T), respectively. When two therapeutic agents were combined into UCNP-R-T, the synergetic effect increased markedly, ~2200-fold, resulting in IC50 = 0.0024 μg/mL. The combined therapy of UCNP-R-T was demonstrated in vivo. Our recent results on design of targeted agents with important types of the nanoparticles, including luminescent upconversion NPs, photosensitizers, as well as radionuclide and biological toxic principles will be reviewed. The obtained results show promise for effective combined oncotherapy leading to prospective translation to clinical practices.

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Highly sensitive bioanalytical systems based on plasmonic and polymer nanocomposites

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Prospects for application of the plasmon spectroscopy based on metamaterials, which contain dielectric and plasmonic resonators and development of original polymer nanocomposites for highly sensitive electrochemical and optical biosensors and bioanalytical systems based on the nanoparticles of manganese dioxide, the enzyme-polyelectrolyte complexes and gold and silver nanostructures present in this report. The results obtained made it possible to develop a new generation of biosensors, systems for high-performance analysis of enzymatic activity and systems for analysis of multi-component mixtures of enzyme inhibitors, biosensing the most important metabolic parameters, ecotoxicants, bacterial and virus particles.

Overcoming Biological Barriers in Proteins Drug Delivery

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Lectures Abstracts

Fundamental Biocatalysis

Bacteriophage tail spike proteins: Enzymatic interaction with bacterial polysaccharide directs viral specificity

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Keywords: Bacteriophage, polysaccharide, tail spike, deacetylase, depolymerase, protein engineering

Bacterial viruses (bacteriophages) are most abundant living entities that regulate bacterial population. Their application to combat antibiotic-resistant bacterial pathogens is considered as prospective, but requires detailed investigation of bacteriophage biology, particularly the mechanism of primary recognition of the specific bacterial host. Many bacteria that tend to form biofilms (*Enterobacteriaceae*, *Pseudomonas*, *Acinetobacter*) synthesize a thick layer of outer polysaccharides (O-antigens and/or capsular polysaccharide). These molecules are most accessible moieties on the bacterial surface and often serve as a primary receptor for bacteriophages. An interaction occurs via tail spike or tail fiber proteins attached to the baseplate of the phage tail. To provide the penetration of the phage particle to the bacterial membrane through the polysaccharide layer tail spikes possess catalytic domains with polysaccharide-depolymerizing (lyase) or polysaccharide-deacetylating (esterase) function. The structure of such proteins is very uniform independent of phage morphology and specificity. Most abundant folds are trimeric β-helix (Leiman and Molineux, 2008) for lyases and trimeric multidomain α/β motifs (Prokhorov et al., 2017).

The presented work shows that the same type of tail spike protein structure and interaction with bacterial O-polysaccharide is typical for phages infective against phytopathogens from a new family *Pectobacteriaceae* (*Pectobacterium* and *Dickeya* spp.). Using NMR we have determined unique structures of O-polysaccharides of strains representing *Dickeya solani* [→2)-β-D-6-deoxy-D-altrose-(1→] (Kabanova et al., 2019), *Pectobacterium parmentieri* [→3,4)-α-D-Gal*p* (B) →3)-β-D-Gal*p*NAc (C) →4)-β-D-*Gal*p(D) with terminal 1,4 β-D-Man*p*NAc added to (B)]; *Pectobacterium carotovorum* subsp. *brasiliense* [→α–D-Man*p* (B)-(1→3)-α-D-Man*p*-(1→(C) with (1→4) β-D-Man*p*-(1→3)-a-L-Fuc*p* added to (B)], and *Pectobacterium carotovorum* subsp. *carotovorum* [→2)-α-L-Rha*p*-(B)(1→2)-α-L-Rha*p*-(C) (1→2)-α-D-Gal*p*-(D)(1→3)-β-D-Glc*p*NAc(D)-(1→ with (1→3) α-L-6desoxyTal*p* added to (B)]. All these structures are unique among bacteria. Genes encoding tail spike proteins from phages PP35, PP74, PP99, and Q51 specific to corresponding Pectobacterial taxons were cloned and expressed. Recombinant proteins specifically bind bacterial cells and degrade or deacetylate the O-polysaccharide as shown by high-resolution electrospray ionization mass spectrometry.

Acquired data reveal the details of phage-host molecular interaction, and recombinant bacteriophage tail spike proteins may be used for controlled polysaccharide degradation or diagnostic purposes.

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Amicoumacin A kinases: structure, kinetics and function

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Deep functional profiling, antibiotic resistance, genome mining, antibiotic kinase

Exotic sources of microbiota represent an unexplored niche to discover novel microbial secondary metabolites. Previously we applied ultrahigh- throughput (uHT) microfluidic droplet platform for activity profiling of the entire oral microbiome community of the Siberian bear to isolate Bacillus strains demonstrating antimicrobial activity against Staphylococcus aureus. Genome mining allowed us to identify antibiotic amicoumacin A (Ami) as responsible for inhibiting the growth of S. aureus. Proteomics and metabolomics revealed a unique mechanism of Bacillus self-resistance to Ami based on a subtle equilibrium of its deactivation and activation by kinase AmiN and phosphatase AmiO respectively. Here we show detailed description of structural and kinetic analysis of AmiN functionality from two different strains of B.pumilus producers and non-producers of Amicoumacin. AmiN kinase has common features of phosphotraspherases – ATP-binding domain, Brenner's phosphotransferase motif and metal binding site. Hence, we suppose dissociative mechanism of phosphorylation for this enzyme by analogy with aminoglycoside kinases. We found that Ami binding leads to dramatic structural changes resulted in active site closing (Movies). This enzyme closing is a special feature of AmiN and it was not previously observed for aminoglycoside antibiotic kinases. Finally we succeed to show that AmiN kinases poses protein kinase activity. We suppose that AmiN kinases is provided not only protection against amicoumacin antibiotics but also can play unexplored role in B.pumilus cell life.

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Genetic, biochemical and physiological characterization of novel D-amino acid oxidases from *Ogataea parapolymorpha* DL-1

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Keywords D-amino acid oxidase, methylotrophic yeast, Ogataea parapolymorpha, recombinant enzymes, transcriptional regulation, gene knock-out

D-amino acid oxidase (EC 1.4.3.3., DAAO) is FAD- containing flavoprotein catalyzing stereospecific oxidative deamination of various D-amino acids to corresponding alpha-keto-acids and ammonia. DAAO plays important roles in living cells and the enzyme is widely used as biocatalysts in several biotech and biosensor applications.

We have identified 4 new orthologous genes encoding potential DAAOs in the genome of thermotolerant methylotrophic yeast *O.parapolymorpha* DL-1. Transcriptome analysis showed marked differences in their expression in glucose- and methanol- grown cells, effects of induction and repression by different nitrogen sources including D-aminoacid substrates. Gene disruptions, *in situ* enzymatic screens and heterologous expression in *E.coli* strains were used to infer enzymatic, physicochemical and physiological functions of the encoded proteins.

All four studied DAAOs were obtained as soluble functional active recombinant proteins using the original optimized procedure for production of these flavooxidases in *E.coli* cells. Comparative analysis of enzymatic and physicochemical properties of recombinant DAAOs showed marked difference in their substrate specificity, Km, Vmax, Kcat values, pH-and temperature optimum and stability. Thus, recombinant HPODL_02914 is a typical DAAO with increased activity towards small nonpolar aminoacids, recombinant HPODL_2165 prefers substrates with bulky hydrophobic radicals and HPODL_2400 is D-aspartate oxidases active against D-Asp and D-Glu. Differences in substrate specificity are correctly explained by computer modelling of active site of respective enzymes.

Single, double and triple knockout of the studied genes were created using the original *cre-lox* mediated gene disruption systems for *O.parapolymorpha*. While knockout strains showed no or minor defects in growth parameters in the course of cultivation in rich and minimal medium, drastic stimulatory effects of some double knockouts on cellular DAAO activity, gene expression and cell proliferation on the media with D-aminoacids as sole nitrogen source were observed.

Studies are in progress towards identification of molecular mechanisms of the observed effects, detailing of subcellular localization, physiological roles, enzymatic and physico-chemical properties of the *O.parapolymorpha* DAAO/DDO.

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Effect of post-translational modifications on the amyloid transformation of proteins

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Keywords: post-translational modification, glycation, amyloid proteins

Various types of post-translational modification of proteins can both stimulate their aggregation and impede their amyloid transformation. In addition, without sumoylation and ubiquitination, protein degradation by proteasome systems is impossible. Consequently, any modification of amino acid residues leading to the emergence of a label, recognizable by proteasomes, also changes the content of proteins involved in aggregation and amyloidization. In our work, we studied the effect of different types of protein glycation on their aggregation and amyloid transformation. It has been proven that glycation of amyloidogenic proteins, such as the prion protein and alpha-synuclein, stimulates their amorphous aggregation. Moreover, such a modification prevents the formation of fibrillary structures without significant interfering with the formation of beta-folds. In addition, glycation can affect the same lysine residues that are subject to sumoylation and ubiquitination. Consequently, the pathological effect of glycation on amyloidogenic proteins may be due to three factors: stimulation of amyloid transformation (the formation of oligomeric forms of alpha-synuclein and prion protein), slowing the degradation of amyloidogenic proteins by proteasomes, and the prevention of fibrillation.

We also discovered the effect of oxidative stress on the intensification of protein glycation with aldehydes (methylglyoxal and glyceraldehyde-3-phosphate) due to inhibition of the final stages of glycolysis.

A hypothesis about the relationship of glycolysis and glycation with the development of neurodegenerative diseases of amyloid nature is proposed. The features of native and glycated non-amyloid proteins (for example, milk casein) are discussed, as well as protein sulfation as a possible way for regulating protein-protein interactions, different from phosphorylation.

Acknowledgments

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Malic enzymes in the metabolism of obligate methanotrophs

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Keywords: methanotrophs, malic enzyme, *Methylomicrobium alcaliphilum*, *Methylosinus trichosporium*

Aerobic bacteria utilizing methane as an energy and carbon source (methanotrophs) play an important role in the environment mitigating hazardous effect of CH_4 and have increasing potential for biotechnological applications as producers of valuable substances for human use. Two obligate methanotrophs - *Methylosinus trichosporium* OB3b, assimilating methane carbon via the serine pathway and *Methylomicrobium alcaliphilum* 20Z with the ribulose monophosphate (RuMP) cycle are promising bacteria for methane-based technologies and the model organisms for studying on methylotrophic metabolism. In these methanotrophs we studied the biochemical properties of the malic enzyme catalyzing the oxidative decarboxylation of malate to pyruvate. The malic enzyme is a player in the PEP-Pyruvate-Oxaloacetate node responsible for the distribution of the carbon flow between catabolism, anabolism and energy supply in the cell. The recombinant malic enzymes from the two methanotrophs were obtained by heterologous expression of the *mae* genes in *Escherichia coli* and purified by an affinity chromatography.

The malic enzyme from *Ms. trichosporium* (6 x 81 kDa) catalyzed the reversible $NADP^+$-dependent reaction of malate decarboxylation in the presence of K^+ (or NH_4^+) and Mg^{2+} (or Mn^{2+}). The enzyme was most active at 60 °C and pH 7.5 demonstrating the maximal activity of 36 U/mg of protein in the direction of malate decarboxylation and activity of 7 U/mg of protein in the direction of pyruvate carboxylation. The following *K*m values were obtained: for malate - 2.73 mM, $NADP^+$ - 64 μM, pyruvate - 6.02 mM, and NADPH - 47 μM. Enzyme was inhibited by 50 mM Na^+, 1 mM hydroxypyruvate and 0.2 mM acetyl-CoA. The *Ms. trichosporium* malic enzyme contains C-terminal fragment from 322 amino acid residues homologous to phosphoacetyltransferases. The C-domain was not active as phosphoacetyltransferase but was responsible for the correct oligomerization of the protein and determination of inhibitory effect of acetyl-CoA. We suggested that in this methanotroph, the combined work of phosphoenolpyruvate carboxylase, malate dehydrogenase, pyruvate phosphate dikinase and malic enzyme leads to the synthesis of NADPH needed for fatty acids biosynthesis and intracytoplasmic membranes formation.

The malic enzyme from *M. alcaliphilum* (4 x 65 kDa) catalyzed an irreversible reaction of the NAD^+-dependent decarboxylation of malate to pyruvate with activity of 32 U/mg of protein. The reaction was strongly depended from Mg^{2+} or Mn^{2+} whereas Na^+ and NH_4^+ reduced the enzyme activity but K^+ ions did not affected. No other effectors for the enzyme were revealed. The *K*m values for malate and NAD^+ were found to be 5.52 mM and 57 μM respectively. The disruption of *mae* gene was not affected the growth rate but the mutation was resulted in 20-fold increase of intracellular malate content indicating an importance of this reaction of malic acid conversion. In consideration of the high *K*m value for malate we assume that the malic enzyme can return the "excess" malate to the main metabolism of *M. alcaliphilum*. Moreover, conversion of phosphoenolpyruvate into pyruvate via reactions catalyzed by phosphoenolpyruvate carboxykinase, malate dehydrogenase, malic enzyme and pyruvate phosphate dikinase produces two PPi molecules therefore triggering the highly effective PPi-dependent Emden-Meyerhof-Parnas glycolysis.

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Changes in peroxidase activity in horseradish (*Armoracia rusticana)* roots with red light additional irradiation under conditions of aeroponic phytotron.

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Key words: horseradish peroxidase*, Armoracia rusticana,* peroxidase activity, photostimulation.

The horseradish plants, ***Armoracia rusticana***, were grown under conditions of aeroponic phytotron, with programmatically varying lighting, in order to increase the level of peroxidase activity. Grown in aeroponic conditions, the root system of horseradish plants was different from those grown traditionally, in open ground. The increased ramification of small roots and the presence of a whole group of thicker pre-tapered roots was discovered . The distribution of peroxidase activity values over the organs of aeroponic plant specimens differed from the distribution of activity in plants grown in open ground. We have shown that in aeroponic cultivation of horseradish peroxidases for isolation not only from easily accessible roots, but also from petioles is possible to use. The activity of peroxidases in "aeroponic" roots linearly increased with increasing root thickness. Peroxidase activity was determined by the reaction with 3,3 ', 5,5'-tetramethylbenzidine (TMB).
Into a cuvette containing 3 ml of the reaction mixture ($5x10^{-4}$ M H_2O_2, 1 mg / 150 ml TMB, citrate buffer pH = 5.0) the 20 μl of plant extract was added. After a 12-minute incubation in a dark thermostat at 30 ° C .Using a Hach DR / 4000V spectrophotometer at λ = 450 nm the optical density of the sample was measured.
Plants were stationary grown in balanced white light (LED illumination 400 μmol / m^2sek). The study of the reaction of plants to changes in the light spectrum was carried out according to the following procedure. The first step: during the day, in addition to the white spectrum (10% full power), 90% of the power of the blue spectrum (450 nm) was added. Second step: the plants for three hours in addition to the white spectrum (10% full power) with red light (660 nm) 90% of the power were irradiated. At the first and second steps, that is, after 1day of blue and for 3 hours of red irradiation, samples of roots and stems were taken, frozen in liquid nitrogen and no later than a day later, in the entire series, the peroxidase activity was determined. It was found that with an increase in the proportion of red light in the irradiation, there was an increase in the activity of peroxidases - in the roots by 1.8 times and in the petioles by 1.13 times.
Studying the possibility of inducing peroxidase activity by changing the spectral composition of the radiation is a promising way to produce enzymes.

Orbital Factors Affected on Electron Transfer and Light Energy Conversion in Photoenzymes and Model Systems: A Connection between Spin Exchange and Electron Transfer

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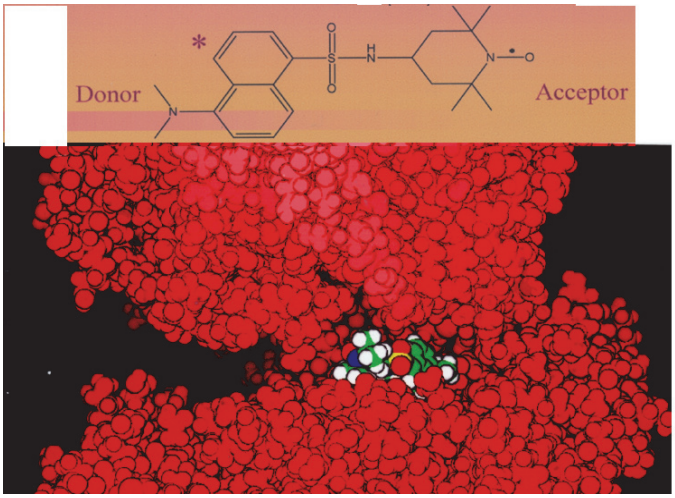
Donor-acceptor structures that retain the charge-photoseparated state long enough for secondary chemestry reactions of these charges to occur are of interest for use of light energy. The aspect of fundamental importance for natural and artificial photosynthesis is the role of orbital effects in the mechanism of conversion of light energy into chemical energy in the primary charge photoseparation. The suggested approach is based on an analogy between superexchange in electron transfer (ET) and such electron exchange (processes as triplet-triplet energy transfer (TTET) and spin-exchange (SE) [1]. This approach allows to estimate values of the exchange integral (I_{SE}) and rate constant of electron transfer (k_{ET}) using experimental data on long distance TTET and SE in bridged nitroxide biradicals. Following this way, we estimated values of I_{SE} and k_{ET} for key stages in a photoenzyme – the bacterial reaction center (BRC). Probing environment with dual fluorophore-nitroxide supermolecules, (FNS) in which fluorophore is tethered with nitroxide, a fluorescence quencher, after incorporated in a protein, opens unique opportunities to quantitative study orbital phenomena, molecular dynamics, micropolarity which affect intramolecular fluorescence quenching, electron transfer, photoreduction and light energy conversion. The estimated values of I_{SE} and k_{ET} for various systems were found to be in good agreement with corresponding experimental data. Suggested equations can be used for calculation of k_{ET} in unknown objects of interest.



Dual probe FNS incorporated in hydrophobic packet of bovin serum albumin

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Regulation of the mTORC1 pathway

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The highly conserved mechanistic Target of Rapamycin Complex 1 (mTORC1) is a protein kinase complex, which controls eukaryotic cell growth and response to a variety of signals, including nutrients, hormones and stresses. The pathways that convey upstream signals to mTORC1 are frequently deregulated by mutations in cancer and other diseases. Moreover a number of proteins that function upstream of TORC1 in the response of different stresses are tumor suppressors. We have recently identified a novel upstream regulator of mTORC1 – the multiprotein SEA/GATOR complex. Several components of GATOR are mutated in different cancers and involved in the resistance to the anti-cancer drugs. I will provide an overview of the upstream regulation of the mTORC1 pathway and its role in cancer, with a special attention to the function of the SEA/GATOR in this process.

Non-protein effectors of the proteasome

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Keywords: proteasome, inhibition, activation

The proteasome-ubiquitin system and its central enzyme, the proteasome, play a key role in regulating the life span of most intracellular proteins. Proteasome is a multisubunit protein complex, the main function of which is the directed and selective degradation of most proteins that have completed their cycle, as well as potentially harmful irregular or unfolded proteins that can be produced during various pathological processes. Each 20S proteasome subparticle contains six proteins with proteolytic activity, two of each of three types: beta1, beta2, and beta5. They possess different substrate specificity, hydrolyzing peptide bond after negatively charged (beta1), positively charged (beta2) or bulky hydrophobic (beta5) amino acids. In some cell types, as well as under the action of pro-inflammatory cytokines, so-called immunoproteasomes can be expressed, with the active centers having partially different substrate specificity, hydrolyzing the peptide bond after small hydrophobic (beta1i), positively charged (beta2i) or hydrophobic (beta5i) amino acids. Most of the known proteasome inhibitors are not very specific, and are able to bind in several active sites, simultaneously stopping hydrolysis after amino acid residues of various types. Therefore, to study the proteolysis of poorly degradable proteins and their fragments that are prone to aggregation, it is necessary to use a whole set of inhibitors to determine which of the active sites are more involved in the process of degradation of such proteins. Besides, there are low-molecular substances that can increase the hydrolytic activity of the proteasome, but very little of them are known. Activation of the proteasome may be one way of treating diseases such as polyglutamine disorders and possibly other proteopathies. In our work, a number of substances were investigated, both known as effectors of the proteasome and new, not previously studied. The dependences of proteasome activity on several substrates on the concentration of these compounds were studied, and the concentrations of IC50 were calculated. Some of them show the ability to stop intracellular proteolysis. Effectors that increase the activity of the proteasome are found among the compounds studied. Such compounds can be used to accelerate the degradation of difficult degradable proteins or protein fragments.

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Functional role of DNMT3a DNA methyltransferase mutations observed in acute myeloid leukemia

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Key words: DNA methylation, Dnmt3a, mutations in leukemia, methylation activity of mutants, interaction with partners, molecular mechanisms.

DNA methylation of cytosine residues in CpG sites is an epigenetic modification that plays an important role in the regulation of gene expression and in other biological processes. In mammals, DNA methylation is introduced by *de novo* DNA methyltransferase (MTase) Dnmt3a. Recently genomic studies on acute myeloid leukemia (AML) have demonstrated that a gene encoding for human DNMT3A (human enzyme is denoted by capital letters) is frequently mutated with striking prevalence of R882H mutation. R882H has been extensively studied and its potential carcinogenic effect has been suggested. We investigate the role of the other missense mutations in DNMT3A catalytic domain found in AML (S714C, R635W, R736H, R771L, P777R, and F752V) using accordingly mutated murine Dnmt3a catalytic domain (Dnmt3a-CD) and 30-mer CpG-containing DNA substrates as model system. Human and murine enzymes have identical primary structures of the catalytic domain. *In vitro* methylation assays showed the 3-5-fold reduced activity for R181L (R771L), S124C (S714C) and P187R (P777R) mutants. The most pronounced reduction of the activty was observed for F152V (F752V), R45W (R635W) and R146H (R736H). Further, the effect of these mutations on individual steps of the methylation reaction was studied. R181L (R771L), S124C (S714C) and P187R (P777R) preserve the ability to bind DNA as it was shown by similar dissociation constants for the Dnmt3a-CD/DNA complexes. In the case of R45W (R635W) and R146H (R736H) a complete loss of DNA binding properties was observed. Finally, the ability of the DNMT3A partner protein DNMT3L to restore the methylation activities of S124C (S714C) and R181L (R771L) was revealed. Hence, mutation in DNMT3A leads to diverse levels of activity and interaction with Dnmt3a partners. Strikingly, all the mutations except S124C (S714C) are not located in the DNMT3A catalytic loop. The contribution of the studied specific residues to molecular mechanism of DNMT3a-mediated DNA methylation was suggested. The role of aberrant DNMT3a activity in AML was discussed on the basis of our knowledge of how these mutations affect methylation function. Collectively, these data together with previously studied R790 and R792 DNMT3a mutants [1] suggest functional impairment of DNMT3a during pathogenesis.

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1. O.V. Lukashevich, N.A. Cherepanova, R.Z. Jurkovska, A.Jeltsch and E.S. Gromova, ***BMC Biochemistry*** (2016) 17:7 (1-10).

Deciphering the functional requirement of zinc finger motif(s) in *Helicobacter pylori* Topoisomerase I catalysis

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Keywords: *Helicobacter pylori*, Topoisomerase I, Zinc finger motifs

Helicobacter pylori, causative agent of gastric cancer, manifests differential gene expression during various stages of stomach colonization. Topoisomerases play a crucial role in maintaining the DNA superhelicity and therefore gene expression. *H. pylori* has only two topoisomerases: Topoisomerase I and DNA gyrase. There is an enormous variability in the C-terminal domain of Topoisomerase I across bacteria. CTD of Topoisomerase I from *H. pylori* (HpTopoI) is unique as it harbours four zinc finger motifs (ZFs) as opposed to three in the widely studied TopoI from *Escherichia coli*. Limited biochemical knowledge of this essential enzyme makes HpTopoI an interesting candidate to study its mechanistic details. Functional characterization of HpTopoI revealed that it binds both ssDNA and dsDNA with similar affinities. Sequential deletion of the third and fourth ZFs had modest effect on the HpTopoI activity. However, combinatorial deletion of second along with third and fourth severely reduced the DNA relaxation activity. Deletion of all ZFs drastically reduced DNA binding and abolished DNA relaxation. Interestingly, our data suggests that CTD alone, comprising of four ZFs has DNA relaxation activity. Furthermore, the highly conserved annotated active site tyrosine residue mutation to phenylalanine (Y297F) did not abrogate the enzyme activity. The analysis of *H.pylori* genomes from 76 strains has revealed presence of multiple copies of genes annotated as TopoI. Most of the homologues within the strain were CTD truncated versions of the Full-length (FL) TopoI. Interestingly, strain XZ274 (isolated from a Tibetan gastric cancer patient) has two chromosomally annotated TopoI genes but lacks the FL version i.e 2211 bp gene. Instead this strain has genes with lengths 314 bp corresponding to ZFs alone and 1934 bp one copy on the chromosome and one on the plasmid annotated as TopoI. Collectively, the results indicate that not all ZFs are dispensable for HpTopoI activity and hints at the presence of an additional/multiple catalytic site(s) in the enzyme.

Bacterial DNA-mismatch repair endonucleases MutL: structure and interaction with molecular partners

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Keywords: MutL endonuclease, mismatch repair

DNA mismatch repair (MMR) system corrects mismatched bases that are generated mainly by DNA replication errors. In the early stages of MMR, MutL endonuclease incises the error-containing strand of the duplex to initiate the downstream excision reaction that is followed by re-synthesis of the strand. In this study, purified MutL from *Rhodobacter sphaeroides* (rsMutL) was shown possess an endonuclease activity. Based on the alignment of 1483 homologs of MutL from bacteria, we evaluated the conserved functional motifs present. The sequence of rsMutL C-domain was shown to contain 5 motifs comprising rsMutL catalytic center responsible for DNA cleavage including metal-binding sites, while 7 conservative motifs related to ATP binding and hydrolysis specific to GHKL-family of ATPases were present in rsMutL N-domain.

The analysis of sequence and biochemical properties for 5 studied homologues of MutL with endonuclease function revealed that MutL from *Neisseria gonorrhoeae* (ngMutL) is most similar to rsMutL. The presence of ATP inhibits DNA cleavage induced by MutL from both organisms. Mg^{2+} promotes the nicking of plasmid DNA by rsMutL with the highest efficiency. The presence of Mg^{2+} and Mn^{2+} in the reaction mixture leads to a significant increase in quantity of linear plasmid form, probably due to the activation of the catalytic center of the second MutL subunit. Zn^{2+} attributed to structural function in MutL homologues was shown to suppress rsMutL and ngMutL endonuclease activity in the presence of excess Mn^{2+}.

The ability of rsMutL and ngMutL to interact with DNA and ATP has been investigated. Complex formation of rsMutL with duplexes of varying length from 4 to 76 base pairs was not observed, while ngMutL formed a complex with a 30-base pair duplex. The K_M values of the ATP hydrolysis reaction by the ngMutL protein are almost two times lower than for rsMutL, indicating a higher affinity of ngMutL to ATP. However, rsMutL hydrolyzed ATP an order of magnitude faster than ngMutL. Perhaps, this causes a large conformational mobility of rsMutL, which does not allow stable formation of a short-lived DNA-protein complex.

The affinity modification of the ngMutL by reactive DNA containing the pyridyldisulfide group demonstrated the proximity of Cys residues of the catalytic center of the enzyme to the DNA ligand.

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Structure and mechanism of D-ornithine/D-lysine decarboxylase, a stereoinverting Fold-III decarboxylase

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Keywords: Pyridoxal-5'-phosphate; Fold III-decarboxylase; Inversion of Stereochemistry, Concerted decarboxylation

STM2360 is a gene located in a small operon of unknown function in *Salmonella enterica* serovar Typhimurium. We have found that the protein coded by STM2360 has a previously unknown catalytic activity, D-ornithine/D-lysine decarboxylase (DOKDC) (1). The substrate specificity is D-Orn ~ D-Lys > D-Arg. The physiological substrate of DOKDC is likely to be D-ornithine, since STM2358, located in the same operon, is an ornithine racemase (1). This is only the second known PLP-dependent decarboxylase acting on a D-stereocenter (diaminopimelate decarboxylase (DAPDC) acts on a *meso* substrate), and the first to act on D-amino acids. The x-ray crystal structure of DOKDC has been determined to 1.72 Å (2). DOKDC has low sequence identity (<30%) with DAPDC and L-lysine/ornithine decarboxylase (LODC), but the three dimensional structure is very similar. However, this enzyme does not exhibit DAPDC activity. The distal binding site of DAPDC contains a conserved arginine that forms an ion pair with the L-carboxylate end of DAP. In both LODC and DOKDC, this distal site is modified by replacement of the arginine with aspartate, changing the substrate specificity. L-Ornithine decarboxylase (ODC) and LODC have a conserved phenylalanine on the *re*-face of the PLP-complex that has been found to play a key role in the decarboxylation mechanism by introducing electrostatic destabilization. We have found that both DAPDC and DOKDC have tyrosine instead of phenylalanine at this position, which precludes the binding of L-amino acid substrates. Since the PLP-binding lysine in ODC, LODC, DAPDC, and DOKDC is located on the *re*-face of the PLP, we propose that this is the acid group responsible for protonation of the product, thus resulting in the observed retention of configuration for decarboxylation of L-amino acids by ODC and LODC and inversion of configuration for decarboxylation of D-stereocenters by DAPDC and DOKDC. The reactions of DAPDC and DOKDC are likely accelerated by positive electrostatics on the *re*-face by the lysine ε-ammonium ion, and on the *si*-face by closure of the lid over the active site, resulting in desolvation and destabilization of the D-amino acid carboxylate.

1. "STM2360 Encodes a Novel D-Ornithine/D-Lysine Decarboxylase in Salmonella enterica serovar typhimurium", Phillips, R. S. Poteh, P., Miller, K. A. and Hoover, T. R., *Arch. Biochem. Biophys*, 634, 83-87 (2017).

2."The crystal structure of D-ornithine/D-lysine decarboxylase, a stereoinverting decarboxylase: Implications for substrate specificity and stereospecificity of fold III decarboxylases", Phillips, R. S., Poteh, P., Krajcovic, D., Miller, K. A. and Hoover, T. R., *Biochemistry*, 58, 1038–1042 (2019).

Application of X-ray crystallography, comparative modeling and molecular dynamics to investigation of structural determinants of enzymatic activation and substrate specificity

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Keywords: transaminase from *H. ochraceum*, oligopeptidase B from *S. proteomaculans*

The X-ray crystallography is widely used for clarification of the structural factors determining the substrate specificities and catalytical mechanisms of enzymes, but it is time consuming, expensive and sometimes limited by the features of a particular target protein. In the absence of an experimentally determined structure, comparative or homology modeling, which uses experimentally determined protein structures to predict the conformation of other proteins with similar amino acid sequences, can provide a useful 3D model. The average accuracy of these models based on more than 50% sequence identity approaches that of low resolution X-ray structures (3Å resolution).

Macromolecules undergo changes with time and condition thereby affecting the structural and functional properties. Existence of multiple conformational states is an example. Obviously, both X-ray and comparative modeling provides a snapshot, which captured just one from numerous sequential variations of a spatial structure. The conformational dynamics of a biomolecule is definitely crucial to understanding its biological functions. Molecular dynamics (MD) simulations are used to investigate dynamics and interactions of proteins in aqueous solution. MD is a form of computer simulation in which atoms and molecules are allowed to interact for a period of time under known laws of physics, giving a view of the motion of the atoms. Molecular dynamics probes the relationship between molecular structure, movement and function.

In this work either X-ray crystallography or comparative modeling and molecular dynamics were incorporated in structure-functional investigation of two different model enzymes: PLP fold type IV transaminase with a mixed type of activity from myxobacterium *H. ochraceum*, and oligopeptidase B from psychrophilic gamma-proteobacteria *S. proteamaculans*. Pyridoxal-5'-phosphate (PLP)-dependent transaminases are industrially important enzymes catalyzing the stereoselective amination of ketones and keto acids. Transaminases with a mixed type of activity were recently identified among PLP fold type IV transaminases as enzymes with abilities to both (*R*)- and (*S*)-stereoselective transfer of amino groups. Oligopeptidases B (OpBs) are serine peptidases with trypsin-like specificity belonging to the family of prolyl oligopeptidases (POPs), all POPs have a characteristic N-terminal β-propeller domain that prevents large substrates entering into the active center located in the interface with α/β-hydrolase domain. OpBs are known to be virulence factors of severe protozoan and, presumably, bacterial infections, and target for drug designing.

The work was supported by the RFBR, project № 18-04-00748 (in part of transaminase from *H. ochraceum* expression, purification and structural analysis) and the RSCF, project № 17-14-01256 (in part of oligopeptidase B *from S. proteomaculans* structure-dynamical study).

3D structure of the natural tetrameric form of human butyrylcholinesterase as revealed by cryoEM, MD and SAXS

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Keywords: butyrylcholinesterase; tetramer; cryoEM; molecular dynamics; 3D structure

Human plasma butyrylcholinesterase (hBChE, EC 3.1.1.8) is an endogenous bioscavenger that hydrolyzes numerous medicamentous and poisonous esters and scavenges potent organophosphorus nerve agents. Thus, hBChE can be used to protect acetylcholinesterase as well as a marker for diagnosis of OP poisoning. It is also considered as a therapeutic target against Alzheimer's disease. Though the X-ray structure of a partially deglycosylated monomer of human hBChE was solved 15 years ago, all attempts to determine the 3D structure of the natural full-length glycosylated tetrameric human hBChE were unsuccessful so far.

Here, a combination of three complementary structural methods – single particle cryo-electron microscopy, molecular dynamic simulations and small-angle X-ray scattering were implemented to elucidate the overall structural and spatial organization of the natural tetrameric human plasma hBChE. A 7.6 Å cryoEM map clearly shows the structural organization of the enzyme: a dimer of dimers with a non-planar monomer arrangement in which the interconnecting super helix complex PRAD-(WAT)4-peptide C-terminal tail is located in the center of the tetramer, nearly perpendicular to its plane and deeply plunged between the four subunits. Time-averaged molecular dynamics trajectories allowed to optimize the geometry of the molecule and to reconstruct structural features obscured in the cryoEM density, e.g glycan chains and glycan inter-dimer contact areas as well as inter-monomer disulfide bridges at the C-terminal tail. Finally, SAXS data confirmed the consistency of the obtained model with the experimental data.

This work was supported by the Russian Science Foundation projects - #19-14-00164 (in part of the data collection and analysis) and #17-14-01097 (in part of the sample preparation) as well as by Russian Foundation for Basic Research project #19-03-00043 (in part of molecular dynamics simulation).

Sulfoxides of sulfur-containing amino acids - suicide substrates of methionine gamma-lyase as antimicrobial and antimycotic prodrugs.

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Keywords: methionine gamma-lyase, suicide substrates, thiosulfinates, prodrugs.

Pyridoxal 5'-phosphate-dependent methionine γ-lyase (EC 4.4.1.11, MGL) catalyzes the γ-elimination reaction of L-methionine and its analogues, as well as the β-elimination reaction of L-cysteine and S-substituted L-cysteine to yield ammonia, α-keto acids and thiols.

Sulfoxides of the L-methionine, L-homocysteine, and S-alk(en)yl-L-cysteine are suicide substrates of the enzyme. The products of the β- and γ-elimination reactions of sulfoxides, thiosulfinates inactivate MGL by the oxidation of cysteine residues.

Three-dimensional structures of inactivated by dimethyltiosulfinate and diethylthiosulfinate MGL were determined at 1.8 Å and 1.6 Å resolution. Analysis of the structures revealed structural bases of MGL inactivation by thiosulfinates.

Mutant form of the enzyme with the replacement of active site C115 for H (C115H MGL) cleaves (±)-S-alk(en)yl-L-cysteine sulfoxides more effectively than the wild type enzyme does.

In vitro the "pharmacological pairs" C115H MGL + sulfoxides of S-substituted-L-cysteine demonstrated growth inhibition of Gram-positive, Gram-negative bacteria, multidrug-resistant strains of *Achromobacter ruhlandii* 155B, *Burkholderia cenocepacia* 122 and *Pseudomonas aeruginosa* 48B isolated from patients with cystic fibrosis, and antimicotic activity against *Candida albicans* ATCC 24433 strain and clinical isolates of *C. albicans* from patients with cystic fibrosis.

In vivo pharmacological pairs combined with antibiotics demonstrated synergetic or additive effects on *C. albicans* strains.

C115H MGL encapsulated in polyionic polymeric vesicles (C115H-PICsomes) provides 100 % survival of mice infected by *A. ruhlandii* 44B and *S. aureus* 129B strains. The therapeutic efficacy against infection caused by *P. aeruginosa* was observed for only the pair C115H-PICsomes + sulfoxide of S-methyl-L-cysteine.

The work was supported by the Russian Science Foundation (project # 15-14-00009).

Effects of amino acid substitutions in the characteristic sequence motifs on the profile of substrate specificity of PLP fold type IV transaminases

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Keywords: enzyme catalysis, substrate specificity, transaminases, bioinformatics analysis, 3D structure

Transaminases are one of the most promising tools in organic synthesis as biocatalysts of stereoselective transfer of amino group on ketones and ketoacids. The search for the effective transaminases initiated the development of approaches to identify substrate specificity of transaminases based on the analysis of their amino acid sequences. Good *in silico* annotation algorithm was developed for PLP-dependent transaminases of fold type IV by Höhne et al (Nature Chem. Biol. 6 (2010), 807–813). The authors inferred from protein topologies, crystal structures, and biochemical information characteristic sequence motifs from D-amino acid transferase (DAAT), 4-amino-4-deoxychorismate lyase (ADCL), L-branched chain amino acid aminotransferase (BCAT) and (R)-amine specific transaminase. An *in silico* algorithm led to the identification of 21 putative (R)-transaminases from approximately 6000 candidates. Following their lead, we performed a search for PLP fold type IV transaminases (TAs) with mixed type activity in genomes of archaea and bacteria. We accomplished phylogenomic analysis based on the proteins from COG0115 (IlvE branched-chain amino acid aminotransferase/4-amino-4-deoxychorismate lyase in the COG database Galperin et al. (2015) Nucleic Acids Res 43: D261-269). Five groups of TAs of PLP fold type IV with changes in the substrate specificity-determining sequence motifs were revealed. These sequences are distinguished from the canonical BCATs, DAAT and (R)-amine:pyruvate TAs by substitutions in the active site positions that are important for the substrate recognition.

Codon-optimized sequences for the expression of new TAs from the group I in *E. coli* were synthesized (ATG Service Gene, St. Petersburg, Russia). The recombinant TAs were expressed and purified to homogeneity. We focused on the study of new TAs using the methods of enzyme kinetics, X-ray crystallography, and molecular modeling. The effects of amino acid substitutions in the characteristic sequence motifs on the profile of substrate specificity of new TAs were estimated. This study has shown that multiple substrate preferences toward both various L-amino acids and (R)-primary amines can be implemented within one PLP-dependent active site of the fold-type IV.

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Investigation of T2 channel of two-domain laccases

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1nstitute of Protein Research RAS

Keywords: two-domain laccases, crystal structures, T2/T3 copper site, channels

Laccases (EC 1.10.3.2) are blue multicopper oxidoreductases acting on diphenols and related substances so far found in higher plants, some insects, fungi and bacteria. Laccases are highly important for biotechnology and environmental remediation. These enzymes contain four Cu atoms per molecule organized into three sites: T1, T2 and T3. Mononuclear T2 copper ion and two T3 copper ions form a trinuclear center (TNC). T1 acts as center for the reception of the electrons from the reducing substrate, while the TNC serves as binding site for molecular oxygen and its reduction to water. The channel leading towards the Cu2 (T2 channel) can be responsible for the transport of protons to the active center and removal of the reaction product (water).

Along with the typical three domain laccases bacteria produce two-domain laccases (2DLac) which are functionally active in the neutral and alkaline pH area, thermostable and stable against inhibitors. The catalytic mechanism of 2DLac has been intensively studied, but substrate/product transport to/from TNC is not yet fully understood. T2 and T3 channels in 2DLac are less accessible than three domain laccases, in these structures the chains of water molecules are interrupted by side chains of amino acid residues, which apparently can play the role of a "gateway".

We present the comparative analysis of crystal structures and catalytic properties of recombinant 2DLac from *Streptomyces griseoflavus* Ac-993 (SgfSL) and two mutant forms with replacements of the conservative Arg240, located in the T2 solvent channel. The side chain of Arg240 is fixed by two hydrogen bonds and these interactions very narrow the potential T2 channel. We applied mutation analysis for accessibility investigations of TNC copper centre via T2 channel. It was constructed by site-directed mutagenesis method and purified mutant forms of SgfSL with replacement Arg240 to His and Ala. We solved crystal structures of SgfSL mutant forms with high resolution (<2 Å) and carried out a comparative analysis with the structure of the wild-type protein. The structures of mutants confirm single amino acid replacements at the desired position, no significant changes in the overall fold of the enzyme or near active sites were observed. This fact made it possible to reliably compare kinetic activity of the mutants to the SgfSL.

The substitutions of Arg240 to an alanine result in decrease catalytic activity at ABTS oxidation by about an order of magnitude. This indicates the important role of the charged residue in the T2 channel.

The substitution of Arg240 to histidine do not changed catalytic activity of mutant form laccase. We suggested that side chain Arg240 located in T2 channel participates in proton transfer of 2DLac. Replacement of arginine with histidine still allows proton transfer, and replacement with alanine does not. In three-domain laccases the role of transfer of protons to TNC plays an acidic amino acid residue located in T2-channel- Asp116 (Silva et.al., 2012).

Thus, we have shown that arginine 240 can play an important role in the protonation mechanisms in two-domain laccases when oxygen is reduced to water.

C.Silva, J.Damas, Z.Chen, V.Brissos, L.Martns, C.Soares, P.Lidley, I. Bento. The role of Asp116 in the reductive cleavage of dioxigen to water in CotA laccase:assistance during the proton-transfer mechanism., Acta Cryst. (2012), D68, 186-193.

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Quantum-chemical analysis of the catalytic reaction of aspartate aminotransferase

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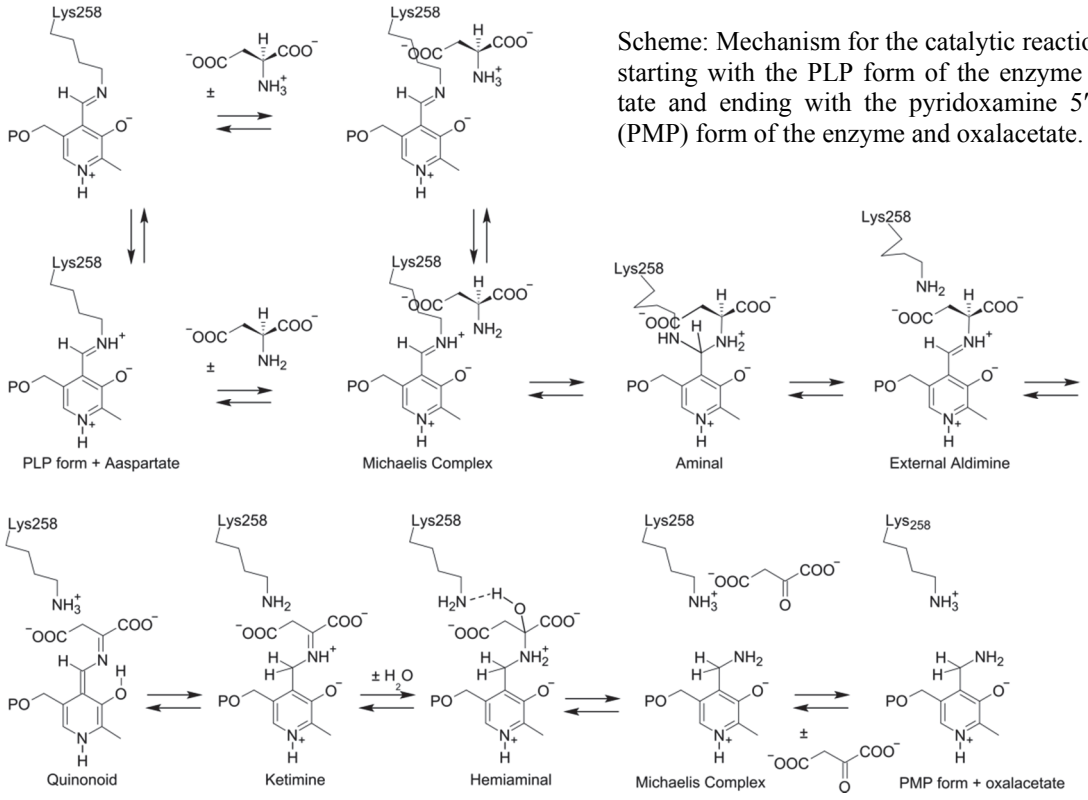
Keywords: pyridoxal 5′-phosphate, aspartate aminotransferase, reaction mechanism, quantum chemistry, concerted mechanism

The reaction mechanism of aspartate aminotransferase (AAT) has been extensively The reaction mechanism of aspartate aminotransferase (AAT) has been extensively studied since its discovery. However, there are still ambiguities about the mechanism—for example, the transaldimination and ketimine hydrolysis steps, both of which involve the formation/cleavage of the imine bonds between PLP and substrates. In order to obtain deeper insights into the reaction mechanism of AAT, we carried out mixed quantum-mechanical/molecular mechanical calculations (B3LYP/6-31G* and Amber 95 force field) on the reaction of AAT with aspartate, based on the model constructed from a 1.2 Å X-ray structure of an *E. coli* AAT–succinate complex.

The results showed that the strain on the PLP–Lys258 aldimine is released in the "external aldimine" of PLP–aspartate, promoting the formation of this complex. However, the precise mechanism of this "transaldimination" process was found to be elusive: in the aminal, both the direct transfer of the proton from Nα of aspartate to Nε of Lys258 and the proton transfer via the phenolate O3′ faced a high energy barrier. The most plausible pathway was the concerted proton transfer involving the phenolic hydroxyl group of Tyr225. The ketimine hydrolysis involves complicated proton transfer steps. Here, the ε-amino group of Lys258 was found to assist the movement by functioning as: the general base catalyst for the attack of a water molecule to the ketimine, proton donor to Nα of the hemiaminal, and finally the base catalyst for the elimination of oxalacetate from hemiaminal.



Scheme: Mechanism for the catalytic reaction of AAT, starting with the PLP form of the enzyme and aspartate and ending with the pyridoxamine 5′-phosphate (PMP) form of the enzyme and oxalacetate.

Origins of enzyme catalytic activity explored within theory of intermolecular interactions and biocatalyst design using catalytic fields

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Keywords: theozymes, intermolecular interactions, biocatalyst design, atomic multipoles

Recent progress in theoretical biocatalyst design has been stalled after some initial successes[1], as theozymes displayed rather low catalytic activity and conventional theories are not yet capable to explain the role of additional mutations in second coordination sphere, introduced by directed evolution experiments[2]. These problems could be resolved by employing differential transition state stabilization (DTSS) approach[3-4] and hybrid variation-perturbation (HVPT)[4] partitioning of intermolecular interaction energy into components defining hierarchy of approximate models of enzyme catalytic activity.

Analysis of several enzyme systems indicates clearly the key role of multipolar electrostatic term[4-6], which has been recently confirmed by experiment[7]. Application of this term allowed us to estimate at low computational cost catalytic activity changes in ketosteroid isomerase[6] and Kemp eliminase[8] resulting from aminoacid mutations[6].

Wherever electrostatic contribution is dominant, it is possible to derive from atomic multipoles of transition states and substrates general characteristics of optimal catalyst in the form of catalytic field[3-4], which represents solution of the inverse catalysis problem. This permitted us to examine dynamic catalytic effects due to rotations of charged aminoacid sidechains explaining role of mutations in the second coordination sphere and to design new mutations leading to enhanced catalytic activity[8]. Catalytic fields fields could be also employed to predict substrate substituent effects on activation energy changes [9], verify alternative enzyme reaction mechanisms and to explore possible catalytic role of proton transfers in hydrogen bond chains present in enzymes.

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Towards deep insight into the cephalosporin hydrolysis in the active site of metallo-beta-lactamase

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Keywords: metallo-beta-lactamase, cephalosporins, drug resistance, QM/MM, QTAIM

The emerging problem nowadays is the increasing resistance of bacteria to many of the existing antibiotics. One of the reasons is hydrolysis of antibiotics by metallo-beta-lactamases as the reaction product cannot inhibit a target protein – penicillin binding peptide. Deep understanding of the details of the reaction mechanism in the active site is required for further analysis of the reasons of hydrolysis of certain antibiotics and resistance of others. Therefore, we present a new interdisciplinary approach combining methods applied for modeling large biological systems and modern methodology from precise analysis of the electronic structure and reactivity of small molecules.

During the cephalosporin hydrolysis in the active site of metallo-beta-lactamase a relatively stable intermediate is formed, and its further decomposition to reaction products is a limiting step. We analyze a set of 10 cephalosporin compounds with the known catalytic rate constant. At the first step, the QM/MM method at the DFT(PBE0-D3/6-31**)/AMBER level of theory is used to construct the equilibrium geometry configurations of protein complexes with stable intermediates of hydrolysis as well as the transition state of the limiting stage. Next, we performed detailed analysis of the electron density in the active site of protein and evaluated a set of electron-density based descriptors. This led us to concept of the key interatomic interactions that mainly describe the differences between the systems under consideration and determine their catalytic activities. In our particular case, those are the properties of the hydrogen bond of the limiting stage. We propose the relationships between these descriptors and macroscopic parameters, catalytic rate constants, that can be utilized in search of novel prospective cephalosporin compounds.

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Towards application of deep convolutional neural networks to enzyme design

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Keywords: computational enzymology, machine learning, enzyme design, QM/MM modeling

Computational enzyme design is an ambitious but still not overcomed challenge. Recently massive combinatorial virtual screening of single point mutations successfully yielded a variant of Ig scavenger with performance increased by two orders of magnitude. However, scaling this approach to even double mutations is unfeasible because of exponentially growing combinatorial space. One of the possible approaches to address this issue is by dissecting the internal logic in the data from single mutagenesis study and then applying it to predict the effect of multiple simultaneous mutations on enzyme activity without explicitly calculating it. Methods of machine learning are perfectly suited for this task. Though some efforts were made to predict enzyme activity based on its sequence, enzymatic activity is directly manifested on the level of structure with crucial role of electrostatics. That is why we believe that structure-based descriptor is more suited for this task. What is more, it acts on the level of chemistry and is agnostic of sequence and alphabet, thus allowing to capture effects of various cofactors such as metal ions and to study non-homologous enzymes with similar function.

In this work we intended to test whether modern architectures of deep convolutional neural networks (CNNs) can be used to correctly predict mutation effect on enzyme's catalytic activity utilizing only 3D structures as input data. Unfortunately, to date there is no large enough dataset linking specific enzyme variants' structure and activity; thus we generated 192 variants of diisopropylfluorophosphatase. This enzyme was selected as a model system because corresponding reaction comprises only one chemical step and is well-studied. Single amino acid substitutions were introduced to all positions in 12Å radius from the active center. For each variant we computed reaction barrier with the help of hybrid QM/MM metadynamics.

Machine learning was performed with recently developed unusual spherical CNN architecture, and proteins' structures were represented as a grid of voxels comprising information about all atoms' radius, charge and mass.

We achieved accuracy over 60% in the three-class classification test (worse, unchanged, better). Though it is only the initial step, we showed applicability of deep spherical CNNs to computational enzyme design.

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Unraveling Na^+ dependent thrombin activation with large-scale QM/MM simulations

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Keywords: thrombin, qm/mm modeling, slow/fast form

Thrombin is the key protease in the blood-coagulation cascade. With more than 500 unique records in the Protein Data Bank (PDB) for human thrombin only - it is one of the most studied enzymes to date. Yet there are several puzzles related to its functioning to be solved. The most intriguing one is the sodium-dependent activation which was discovered in 1980.

Na+-free thrombin has lower activity towards fibrinogen and other coagulation cascade substrates in comparison with the Na+-bound form. Sodium cation binding increases thrombin activity (altering kcat, Km or both in a substrate-dependent manner) up to 10-fold. Hence apo-form of the protein is often referred to as “slow” form, while the holo-form is called “fast”. Under physiologic conditions there is an equilibrium between three states:

$$E^* \square E \square E{:}Na^+$$

where E* is a low-activity form incapable of Na^+ binding, E and $E{:}Na^+$ (Na^+-stabilized form of E) have higher activity.

It is believed that the E form has a definite structure, while E* is represented by an ensemble of conformations. As a consequence of the ensemble heterogeneity, structural studies of the E* form proved to be more challenging than studies of the E:Na+ form. Most of the documented structures of “slow” thrombin contain mutations in proximity of Na+-binding site and crystal packing interactions that might affect conformations of the active site and Na+-binding site.

In this work, we performed large-scale hybrid QM/MM simulations with an unprecedentedly large QM part of 61 amino acid residues and 29 water molecules producing 459 atoms as well as classical MM simulations of thrombin-substrate complexes. We identified several previously undescribed or unattributed structural features of the E* form related to the configuration of the catalytic Ser195. The occurrence of the newly discovered states was confirmed with an analysis of human thrombin crystal structures from the PDB.

Our results reveal detailed picture of the E* form ensemble. We also for the first time demonstrate gradual effects of K+ and Na+ cations on the equilibria shift. Finally, our results clarify some controversies in the interpretation of existing crystal structures of “slow” and “fast” thrombin structures.

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L,D-peptidases of coliphages: structure, properties, regulation and biotechnological potential

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Keywords: L-alanyl-D-glutamate peptidases, bacteriophage, host cell lysis regulation

Lytic bacteriophages at the final stage of their life cycle induce the synthesis of several proteins that destroy the host cell wall allowing the release of phage progeny. One of the key proteins of such a lytic system is an enzyme named endolysin. This is a hydrolase (less often lyase) which disrupts the peptidoglycan, a main component of the bacterial cell wall, containing non-canonical amino acids in its peptide subunit. Particular interest to endolysins is connected with their potential use as antibacterial agents that can serve as an alternative to antibiotics in the treating of bacterial infections.

We have identified and characterized the endolysins of coliphages T5 (Siphoviridae), RB43 and RB49 (pseudo T-even Myoviridae). The genes were overexpressed in pET/BL21(DE3) system, the target proteins were purified to homogeneity. These enzymes are homologous proteins with a molecular mass of 14.7–15.3 kDa. They were demonstrated to hydrolyze the bond between L-alanine and the D-glutamate of the type A peptidoglycan peptide subunit. All enzymes are zinc-containing peptidases belonging to M15 family and differing in their enzymatic properties: specific activity, pH-optimum, sensitivity to ionic strength and buffer compounds. The pH optimum of functioning was found to be within the range of 7.0-9.0, while the optimal values of ionic strength were different: 25 mM for the RB43 and T5 and 100 mM for the RB49. Inhibition of the bacteriophage T5 enzyme by divalent ion chelators and its activation by calcium ions were shown. Using site-directed mutagenesis and the determination of dissociation constants, it was established that the calcium ion binds with a non-canonical EF-like loop with a low affinity — Kd=0.21 ± 0.01 mM. Calcium activation adds another regulation level to the phage-mediated host cell lysis process.

The spatial structure of bacteriophage T5 peptidase in solution was established by high resolution NMR. It was shown that it is a globular protein belonging to the α+β class; the molecule has a hydrophobic core formed by three α-helices and four anti-parallel β-folds forming the β-sheet. The zinc ion in the enzyme structure is coordinated by the conservative amino acid residues H66, D73 and H133; the aspartate residue 130 is catalytic. The structure of the protein was also shown to contain two relatively long flexible disordered loops one of them capable of binding paramagnetic ions.

All characterized enzymes exhibit resistance to high temperature, retaining from 25 to 65% of lytic activity after a half-hour heating at 90°C. This resistance is due to the ability of proteins to renature their secondary structure.

Phylogenetic studies have shown that orthologous sequences are widely represented in the genomes of lytic phages that primarily infect Gram-negative hosts. We can assume a significant role of the horizontal gene transfer between unrelated phages having a common host.

A study of the spectrum of the bacteriolytic action has shown that the enzymes effectively lyse in vitro Gram-positive bacteria of the *Bacillus* group, as well as Gram-negative bacteria of the genera *Escherichia* and *Pseudomonas* (in the presence of agents permeabilizing their outer membrane). The ability to destroy bacterial cells, thermal resistance, conformational stability and high specific activity of these L,D-peptidases make them candidates for application in biotechnology and biomedicine.

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Applied Biocatalysis

Thermophilic Enzymes for Industrial Biocatalysis

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Keywords:Thermophilic enzymes, Structure and Mechanism, Applications for Biotechnology

The stability of enzymes for industrial reactions is often their stability under the conditions employed. Naturally thermostable enzymes isolated from 'Hot' environments are more stable to high temperatures, extremes of pH and exposure to organic solvents [1-3]. The projects **HOTZYME** and **THERMOGENE** have identified hydrolase and transferase enzymes of industrial interest isolated from high temperature environments around the world. A selection of these novel thermostable enzymes including cellulases [4], carboxylesterases [5-7], lactonases, epoxide hydrolases [8,9], transketolases [10], hydroxymethyl transferases and transaminases [11-12] have been characterized both biochemically and structurally.

Transaminase enzymes have received special attention for the production of chiral amines which are important building blocks for the pharmaceutical industries. These enzymes catalyse the reversible transfer of an amino group from a donor substrate onto a ketone/aldehyde or sugar acceptor molecule. They can be subdivided into 6 different classes. Thermophilic examples of the less studied class 4 (BCAT, *R*-selective), class 5 (*S*-selective) and class 6 (sugar) transaminase have been identified including the archaeal class 4 transaminase from *Archaeoglobus fulgidus*; a class 5 archaeal transaminase from *Sulfolobus solfataricus* and two different class 6 sugar transaminases in the archaea *A. fulgidus* and in the bacteria *Thermovirgo* species.

Transketolase enzymes are able to form a new carbon-carbon bond and transfer a 2-carbon unit to an acceptor sugar in the pentose phosphate pathway. The reaction can be made irreversible using β-hydroxypyruvate as the 2-carbon ketol donor and glycoaldehyde as an acceptor with the production of L-erythrulose and carbon dioxide which is released from the reaction. Thermophilic archaea and deep branching bacteria have both novel 'split' transketolase genes and full length genes. Several novel transketolase enzymes have been cloned, over-expressed and characterised. The structure of one full length thermophilic bacterial enzyme has been determined and one novel 'split' archaeal transketolase. These have been compared with the *E.coli* transketolase and other members of the transketolase family with a view to using the cheaper starting substrate, pyruvate rather than hydroxypyruvate.

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Lytic polysaccharide monooxygenases: structure, mechanism and function

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Keywords: Lytic polysaccharide monooxygenase, catalytic mechanism, oxygen consumption, cellulase, synergism

Lytic polysaccharide monooxygenases (LPMOs) are metal-dependent enzymes that catalyze the oxidative cleavage of various polysaccharides. The oxidative nature of LPMOs has been discovered in 2010-2011, although these enzymes had previously been known as proteins of the CBM33 and GH61 families in the Carbohydrate Active Enzymes (CAZy) database. LPMOs preferentially acting on cellulose are recently reclassified into families 9 and 16 of Auxiliary Activities (AA9 and AA16) in the CAZy database, while those acting on other polysaccharides (chitin, xylans, starch, etc.) are the members of the AA10, AA11, AA13, AA14 and AA15 families. All known LPMOs share similar structure of the active site that contains a copper ion coordinated by the histidine brace; the first of two conserved histidines represents the N-terminal residue of the mature protein. LPMOs cleave a polysaccharide chain with oxidation occurring either at C1 or C4 atom of the glycoside ring with formation of aldonic acid or 4-keto-aldose, respectively; the third group of these enzymes generate products oxidized at both C1 and C4 position. In the first studies, it was believed that molecular oxygen is involved in the formation of the reactive intermediate with copper. More recently, hydrogen peroxide, rather than O_2, has been suggested to be a true co-substrate for LPMOs, while the latest studies indicate that both O_2 and H_2O_2 can be utilized by LPMOs as co-substrates. Cellulose-oxidizing LPMOs have attracted the attention of researchers as enhancers of hydrolytic performance of cellulases in the enzymatic saccharification of cellulose, a key step in the growing biotechnology for production of second-generation biofuels and chemicals from renewable lignocellulosic biomass.

The characteristic features of AA9 family LPMOs from fungi *Trichoderma reesei*, *Thielavia terrestris*, *Myceliophthora thermophila* and *Penicillium verruculosum* will be discussed in this report, covering such properties as the enzyme activity based on the recently developed highly-sensitive fluorimetric assay of the oxygen consumption rate or an alternative assay based on using H_2O_2 as a co-substrate; the influence of pH and effectors on the LPMO activity; enzyme thermostability; a synergism between LPMOs and individual cellulases or their multienzyme cocktails in saccharification of cellulosic and lignocellulosic substrates. The role of a cellulose-binding module (CBM) in LPMO functioning will also be discussed, and the CBM effects on the activity and substrate specificity of a chimeric LPMO, obtained using a protein engineering technique, will be demonstrated.

Novel enzymes for biosynthesis and biotransformation of adipic acid

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At present, the most thermodynamically feasible pathway for adipic acid production is shikimate pathway. Adipic acid is one of the most important aliphatic dicarboxylic acids, which is used for the synthesis of nylon-6-6 and also can be utilized for further biotransformation to 1,6-hexendiol and hexamethelendiamine. Here we focused on several important groups of enzymes participating in the shikimate pathway and also targeting subsequent biotransformation of adipic acid to other industrially valuable compounds (Khusnutdinova et al., 2017). Enzymes we are focused on are protocatechuic acid (PCA) decarboxylase (UbiD family of proteins) which requires prFMN as a cofactor (Wang et al., 2018), that is produced by flavin prenyltransferase (UbiX family of proteins); and 2-enoate reductases. UbiD decarboxylases are important industrial enzymes that are used for vanillin, vine and paper production (White et al., 2015**).** Their activity and application is hindered by prFMN cofactor biosynthesis. We screened more than 20 different UbiX enzymes and identified several FMN prenyltransferases suitable for aerobic activation of UbiD decarboxylases.
Final step of adipic acid synthetic pathway relies on muconic acid double bonds hydrogenation, that previously was performed by non-enzymatic catalysts. Recently, we demonstrated the first enzymatic hydrogenation of muconic acid and 2-hexenedioic acid to adipic acid using bacterial 2-enoate reductases (ERs) (Joo et al., 2017). Based on the structural model of the *Bacillus coagulans* ER (ERBC) over 40 ERBC residues were selected for site-directed mutagenesis, and 29 mutant ERBC proteins were purified and used for *in vitro* activity assay. Presently, based on phylogenetic analysis of Enoate reductase we identified oxygen stable ER applicable for *in vivo* catalysis.
Subsequent biotransformation of adipic acid to 1,6-hexendiol, 6-aminocaproic acid and hexamethelendiamine has been performed using coupled *in vitro* assay with carboxylate reductases, aldehyde reductases and amino transferases. We achieved ~ 5-10% *in vitro* transformation efficiency to hexamethylenediamine, ~ 40% *in vitro* transformation to 6-aminocaproic acid, over 95% *in vitro* and *in vivo* transformation efficiency to 1.6-hexendiol.

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Structure, catalytic mechanism and protein engineering of enzymes Baeyer-Villiger Monooxygenases XanO4 Catalyzes Cyclic Ether Formation

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Biological Bayer-Villiger (BV) oxidation catalyzed by Bayer-Villiger monooxygenases (BVMOs) have been widely used in synthetic biotransformation and typical biological BV oxidations of ketone into ester and lactone have also been displayed in several natural product biosynthesis. However no activity of transformation of ketone into ether has ever been found. Microbial polycyclic xanthone antibiotics features a unique ether-containing xanthone ring, which was speculated derived from anthraquinone precursor catalyzed by BVMOs. We expressed and purified the candidate BVMOs XanO4 encoded in xantholipin biosynthetic gene cluster and we revealed that the type "O" BVMOs XanO4 catalyze an unprecedented oxidative replacement of carbonyl group with an ether oxygen atom unexpectedly accompanied with a demethoxylation in cycloketone, which is currently missing in the inventory of BVMO-mediated reactions. Moreover, genetically and biochemical performance of homologous genes served as assistant support for the generality of the novel BVMO-mediated ether formation. Our work expands the repertoire of BVMOs activities and may benefit synthetic strategy for cycloether intermediates.

Multipoint mutants of TvDAAO with improved catalytic properties and stability

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Keywords: TvDAAO, rational design, oxidation stability, cephalosporin *C*

D-amino acid oxidase (DAAO) catalyzes the conversion of D-amino acids to the corresponding α-ketoacids with formation of hydrogen peroxide and ammonium ion. DAAO plays an important physiological role and is used in various fields of biotechnology. The most important application with DAAO is production of 7-aminocephalosporanic acid (7-ACA) from cephalosporin *C*. 7-ACA is the main synthon for the production of cephalosporin antibiotics of various generations.

To reduce the cost of production using enzymes, it is necessary to improve their properties such as catalytic parameters, temperature stability. In the case of DAAO an important parameter is stability against oxidation of hydrogen peroxide, since one of the reaction products catalyzed by DAAO is hydrogen peroxide. In addition, during 7-ACA production additional hydrogen peroxide is added into the reaction system to complete decarboxylation of the intermediate products. One of the methods to improve these properties is the rational design.

DAAO from the yeast *Trigonopsis variabilis* (TvDAAO) has the highest stability and activity with cephalosporin *C* among all studied DAAOs. In our laboratory we carry out systematic study structure-function relationship in TvDAAO as well as the preparation of new mutant forms of TvDAAO with improved properties using method of directed mutagenesis. We made substitutions witch lead to improvement of temperature stability [1] and increase of resistance against hydrogen peroxide oxidation. Many changes also resulted in improvement of the catalytic parameters in cephalosporin *C* oxidation [2].

In this work positive substitutions were combined and several multipoint mutant forms of TvDAAO were obtained. The best multipoint mutant forms showed more than 4 times higher catalytic constant in the oxidation of cephalosporin *C*, 8-10-fold better stability against hydrogen peroxide oxidation and 20-30 times higher temperature stability compared to wild type TvDAAO.

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Medicine

Use of bacteriophage and lytic enzymes for the detection, treatment and prevention of bacterial infections

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Phages are very small viruses that destroy select bacteria by lysing or by DNA modification. The idea of using phage as a therapy for infectious bacterial diseases was first proposed around WWI and since then has been a key tool within Eastern Europe. More recently lytic and other components have been isolated; and thus further broadened the potential of phage derived technologies with applications for treatments, preventatives, decontaminants and diagnostics.

Although initially focusing on detection and decontamination, preliminary studies through Rockefeller University (NYC, NY) also indicated the phage Lysins may be of used as combinational therapies with antibiotics, humanized monoclonal antibodies and others. The detection system demonstrated sensitivity (~ 100 cell) as well as simplicity. The Pacific Northwest National Laboratory (Richland, WA) also demonstrated effective decontamination utilizing phage lytic enzymes in an "electro-activated" aqueous solution in aerosol (fog) form.

Interest in phage nanobiotechnology, or the use of natural or modified phage or their components is on the rise for use in the development of next generation technology. Use of these enzymes and phage has been further expanded to include replacement or enhancement of antibiotics to treat disease.

The Eliava Institute of Bacteriophage, Tbilisi, Georgia, International Phage Research Center (IPRC) & Jiangsu Academy of Agricultural Sciences (JASS), Nanjing, China and New Horizons Diagnostics Corp (NHD), Maryland, USA and others have teamed together to develop joint projects for the use of these technologies in a broad field of use. The Eliava Institute has been continually developing phage based therapies for almost 100 years. The NHD team developed the first applications utilizing phage lytic enzymes in 1985. IPRC, an International non-profit Institute under the JAAS and the Chinese Ministry of Agriculture , has teamed with Eliava, New Horizons Diagnostics and others to focus on biocontrol as well as an agricultural antibiotic replacement technology. The unique Eliava Staph phage has demonstrated effectiveness against 97% antibiotic resistant strains. The PyC lytic enzyme has been shown to lyse almost 99% of group A Streptococcus within seconds. Other lytic enzymes have been developed for Agricultural use by IPRC/JAAS.

Studies by the Eliava, IPRC/JAAS and NHD utilizing bacteriophage for treatments biocontrol and detection, as well as the use of Phage Lytic enzymes, will be presented with potential opportunities described.

New challenges of contemporary healthcare. Enzyme derivatives of combined action and conjunctive courses of biocatalysts

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Keywords: enzymes, modification of enzymes, superoxide dismutase, catalase, hyaluronidase, combined and course action of medical destination biocatalysts, endothelial glycocalyx.

The application of high-molecular compounds had been became the substantial breakthrough in patient treatment. The enzyme preparations had preoccupied the appreciable place among biopharmaceutical means gradually. The use of enzyme drugs was effective for aims of substitutive therapy (when administered biocatalysts cover the deficit of body enzymes), liquidation of acute injuries (as vascular thrombosis with timely administration of thrombolytic enzymes), for anticancer therapy with enzyme-antibody conjugates (by means of approach ADEPT technology based on application of medicative antibody-direct enzyme prodrug derivative). The research data elicited the observable therapeutic activity of antioxidant enzymes due to blocking and decreasing the injurious action of oxidative stress. In various disturbances of cardiovascular system the oxidative stress is present. It is understand actual role of such mentioned above derivatives for needs of cardiology.

The investigation of antioxidant derivatives (as superoxide dismutase /SOD/ and catalase /CAT/) was perspective according to obtained results especially in conjunctive using. We obtained the covalent bienzyme conjugate consisting of SOD and CAT coupled via glycosaminoglycan of endothelial glycocalyx – chondroitin sulfate (CHS). The latter had contributed the affinity of obtained bienzyme conjugate to injury sites of vascular wall. Earlier it was shown that already during initial stages of atherosclerosis the CHS content was increased in injury sites of vascular wall. Combination of SOD with CAT activity increases the deepness of joint antioxidant action of these enzymes (converting reactive oxygen species to safe products). Besides covalent binding (via CHS) of these enzymes, the simultaneity of combined presence of biocatalysts (in injury centers) is provided. The enhanced efficiency of SOD-CHS-CAT conjugate was demonstrated as compared with effects of various mixtures of enzyme antioxidants. The efficacy of bienzyme conjugate *in vivo* was shown not only for preventive application, but in medicative regime of its administration (i.e. after initiation of oxidative stress development). These data had broadened the potential area of use for SOD-CHS-CAT conjugate.

New requests are appeared with globalization progress. Categories of cardiovascular dangers are altered (there are hire already the effects of weather conditions /abnormal heat, harsh and appreciable nip). This changing picture is specified the emergence of new medicative requests. They manifest in respect to enzyme derivatives in combined application of different classes biocatalysts. The investigation of effects for courses conjunctive therapy is performed for bienzyme SOD-CHS-CAT conjugate with hyaluronidase derivatives against prohypertensive action of angiotensin II and salt load. Noted research aspects demonstrate scientific and medical answers in respect to new challenges of present healthcare.

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Natural scaffolds as modulators of farnesyltransferase activity

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Key words: farnesyltransferase, Alzheimer's disease, Ras proteins, natural scaffolds

Alzheimer's disease (AD) accounts for approximately two thirds of all cases of dementia and the number of patients today is more than 35 million people worldwide. Therefore, the development of effective neuroprotective drugs is considered one of the priorities of modern health care. Recent studies show that post-translational lipid modification of proteins, known as prenylation, may play an important role in the pathogenesis of neurodegenerative diseases [1]. This process is catalyzed by farnesyltransferase (FT) and geranylgeranyltransferase-1 (GGT). Various intracellular proteins, including heterotrimeric G-protein subunits and nuclear lamins, have been prenylated, but the largest and most studied group is the superfamily of the Ras, Rab, and Rho proteins. They regulate a wide range of cellular functions and play a significant role in the pathogenesis of cancer and cardiovascular diseases, cerebrovascular disorders, and are involved in the pathogenesis of neurodegenerative diseases, including AD. FPP and GGPP levels are elevated in the brains of patients with asthma and inhibition of Rho GTPases prenylation reduces beta-amyloid-induced neuroinflammation.

For chemotherapeutic correction of neurodegenerative pathologies, inhibitors of proteins, enzymes, and receptors that regulate signaling pathways can be used. Some of the signaling pathways associated with farnesyltransferase are the Raf / MEK / ERK and phosphoinositol-3-kinase (PI-3K) pathways [2]. These signaling cascades are closely related to Ras proteins. In the present work, the key stages of signaling cascades initiated by Ras proteins are considered, and the processing features of Ras proteins are analyzed. The possibility of their use as promising molecular targets for the development of effective drugs has been shown. Both literary and own data on new strategies for searching for effective farnesyltransferase inhibitors based on natural scaffolds are presented and discussed.

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^{1}H NMR in vivo study of inhibitory neurotransmitter impact to human brain in videostimulation

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The leading role in inhibitory/inflammatory processes play main neurotransmitters: GABA and glutamate. For that reason, the measurement of neurotransmitter concentrations in response to activation is of high interest. A major limitation of the GABA measurement methodology is co-editing of macromolecular signals [1], that may "blur" the effect of neuronal stimulation. In this work we used GABA– MEGA-PRESS pulse sequence for functional MRS (continuous visual stimulation) of GABA without macromolecular signal.

Seventeen healthy subjects (aged 18-29) participated in the study. Philips Achieva dStream 3T and Head-Neck SENSE coil were used. The *InVivo Sensavue* monitor, the mirror and home-made 8 Hz flashing checkerboard video were used for visual stimulation. 3D T1w images were obtained for voxel positioning. The spectroscopy voxel sized 20x40x30 mm was positioned in visual cortex (fig.1). GABA- signal was obtained with MEGA-PRESS sequence: TE=80 ms, TR=2000 ms, NSA=288, MOIST water suppression (WS) and editing pulses applied at δ_{On}=1.5 ppm in ON-series and δ_{Off}=1.9 ppm in OFF-series (9 min 32 s). At first, spectrum in rest was obtained, after that – spectrum during continuous visual stimulation with the same WS and shimming parameters.; GABA-/Cr and Glx/Cr values were found. Home-made program based on the FID-A package was written to compile PRESS spectra with TE=80 from OFF-series. The averaging was done across all 144 dynamics ($PRESS_{tot}$), and across 1-48 ($PRESS_1$), 49-96 ($PRESS_2$) and 97-144 ($PRESS_3$) dynamics, dividing the "PRESS" study into 3 consequent parts for 3 minutes 10 seconds each. PRESS spectra were processed in LCModel. For each participant the relative effect of stimulation (stimulation/rest) on GABA-/Cr, NAA and Glx were found. All relative values were compared with the value = 1 both with Mann-Whitney (MW) criteria.

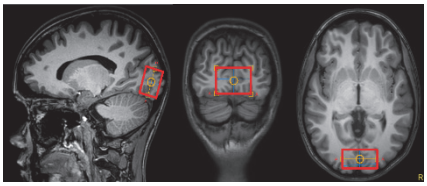

Figure 1. Voxel location in visual

The statistically significant decrease of GABA-/Cr was revealed, Glx/Cr was unchanged. NAA decreased after 9 mins 32 s of visual activation according to $PRESS_{tot}$ processing. Glx increased and NAA decreased after the first 3 mins 10 s ($PRESS_1$); after that both of them remained unchanged (according to $PRESS_2$ and $PRESS_3$)

Table 1. Relative changes (act/rest) of all analysed metabolites (Mean ± Standard Error).

Metabolite	Gannet	$PRESS_{tot}$	$PRESS_1$	$PRESS_2$	$PRESS_3$
GABA-/Cr	0,88±0,09*				
NAA		0,99±0,01*	0,98±0,01**	0,99±0,01	0,99±0,01
Glx	0.99±0,03	0,99±0,01	1,03±0,02**	1.00±0,02	1,01±0,03

- $p<0.05$, ** - $p<0.01$

The decrease of GABA- in current study is in good agreement with [1], where macromolecular fraction is excluded with MEGA-sLASER sequence at 7 T and GABA decreases by 12% during continuous visual stimulation. The growth of Glx in first 3 minutes and the consequent returning back to baseline might be the manifestation of neuronal adaptation.

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Interaction of lysozyme with mucoadhesive polymers

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Keywords: lysozyme, microbial infections, mucoadhesive polymers, multilayer polyion complexes

Lysozyme, the enzyme lysing microbial cells, can be considered as perspective antimicrobial agent. Medicines based on bacteriolytic enzymes should have high antimicrobial activity, be non-toxic, stable in long-term storage. The study aims to develop approaches to optimize the activity/stability of lysozyme for creating novel anti-infectives.

Lysozyme (from chicken egg) has been investigated in its complexes with mucoadhesive and biodegradable polymers (chitosans of molecular weights 150, 400, and 600 kDa and alginic acids of different viscosities) as a basis for creating active and stable antimicrobial. Complexes of lysozyme with polymers were encapsulated by mucin (multilayer polyion complexes were formed). Obtained particles were characterized by physico-chemical and kinetic methods. The data obtained and reasons for observed phenomena are discussed.

Charge-mediated proteasome targeting as a novel type of ubiquitin-independent degrons

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Keywords: proteasome, myelin basic protein, degron, ubiquitin-independent, multiple sclerosis

Ubiquitin-proteasome system (UPS) is known to control metabolism of more than half of the intracellular proteins. UPS consists of approximately 1,000 proteins and many of them are critical for cell functioning and survival. Absolute majority of substrates are recognized by proteasome only being conjugated with ubiquitin (Ub) molecule, representing universal degradation signal operated by ubiquitination system. Ub-independent proteasome targeting is rationalized by existence of two types of direct proteasome signals (DPS) – specific amino acid sequence or posttranslational modification that are recognized by proteasome regulatory subunits. Historically, the first one was shown to be existed in ornithine decarboxylase (ODC), whereas acetylation of core histones was recently reported as second type of DPS. Here we demonstrate the third one, representing charge-mediated DPS. This type of degradation signals, initially discovered in myelin basic protein (MBP) (*Belogurov, Kudriaeva et al. 2014*) and further artificially reconstructed in Basic Elementary Autonomous Degrons (BEADs), is most efficiently engaged by REGα or REGγ-capped proteasomes in ATP-independent manner. Reported DPS may be classified as monopartite element of approximately 100 Å in length enriched in basic, flexible and hydrophobic amino acids. Basic charge was shown to be driving force guiding BEADs-containing proteins to proteasome whereas unfolded state and hydrophobic anchors are rather assisting entrance to the catalytic chamber. The most beneficial position for BEADs is N-terminal location directly upstream targeted protein without any necessity for distal unstructured region, suggesting that topology of substrates containing charge-mediated DPS significantly differs from classical two-component proteasomal degrons. The source of basic charge is not limited to amino acids as bovine serum albumin (BSA) chemically modified by spermine in contrast to glycol-conjugated BSA became ubiquitin-independent proteasomal substrate *(Kudriaeva, Kuzina et al. 2019*). Involvement of REGα in the process of MBP destruction may have important physiological meaning in terms of development of multiple sclerosis. In our previous studies we showed that autoimmune neurodegeneration in central nervous system is tightly linked with ubiquitin-independent MBP proteolysis mediated by immunoproteasomes (*Belogurov, Kuzina et al. 2015*). These immunoproteasomes generate significantly enhanced amounts of MBP-derived immunodominant peptides, which being presented on the surface of oligodendrocytes attract cytotoxic lymphocytes. REGα/β regulatory particles are classical IFN-γ-inducible proteins, directly associated with immunoproteasomes, and involved in antigen presentation in inflammation conditions. Observed here experimental evidences together with our previous results suggest that immunoproteasomes equipped by REGα/β heptamers became deadly machines coordinating autoimmune attack on the myelin sheaths *ab intra*. Study was supported by Russian Science Foundation 19-14-00262.

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Aggregated neutrophil extracellular traps are multifaceted, endogenous biocatalysts

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Neutrophil extracellular traps (NETs) were first described as a mechanism for neutrophils to entrap and kill bacteria. Today, we know that NET formation is not only triggered by bacteria but also by other pathological stimuli such as fungi, viruses and other particulate matter. Another aspect of their anti-inflammatory role is the ability of NETs to shield off necrotic tissue and to close wounds. A plethora of enzymes derived from the granules but also the cytoplasm of neutrophils, such as Neutrophil Elastase (NE), Proteinase 3 (PR3) or Myeloperoxidase (MPO), is associated with the chromatin scaffold of NETs.

High numbers of neutrophils lead to the formation of aggregated NETs (aggNETs) which elicit anti-inflammatory responses by the degradation of inflammatory mediators such as cytokines. These aggNETs further enhance resolution of inflammation by degradation of highly inflammatory molecules such as extracellular histones by actions of NE and PR3. In addition, various glycosidases such as neuraminidase1, β-galactosidase, hexosaminidase A and engase are associated with aggNETs. These sugar-cleaving enzymes are functionally active and are able to modify the glycosylation of Immunoglobulin G (IgG). This potentially results in altered effector function of IgG since the glycosylation is known to be important for these functions. Taken together, the DNA-associated enzymes of aggNETs can be considered an endogenous polyenzymatic biocatalyst.

Activation of antioxidant genetic program: a mechanistic insight

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Keywords: Nrf2, Keap1, bardoxolone, andrographolide, displacement activator

Nrf2 is known as a master regulator of the antioxidant genetic program and triggers the expression of hundreds of proteins and enzymes such as NAD(P)H:quinone oxidoreductase 1, heme oxygenase 1, glutathione transferase, glutamylcysteine ligase and others. In the absence of electrophilic activators or oxidative stress, Nrf2 transcription factor in bound via its regulator protein, Keap1, into the ubiquitin ligase complex Cul3-Rbx1-E3 which catalyzes Nrf2 ubiquitination followed by proteasomal degradation. The biggest interest in the last decade in the field of development of novel Nrf2 activators is focused on non-electrophilic activators working via competitive displacement of Nrf2 from Keap1 complex. Various displacement activators exhibiting the values of dissociation constants in the nanomolar range have been developed in academia and "big pharma" using a homogeneous *in vitro* assay. However, their testing in biological assays and *in vivo* demonstrated a multi-order shift towards the high micromolar range. Taking into account a number of unsuccessful attempts to develop an Nrf2 displacement activator effective in the nanomolar range in the cell-based assays, there is a need in a semi-quantitative analysis (at least) at the cellular level to answer the question on principal limitations for the development of biologically effective Nrf2 displacement activators.

Cell-based reporters expressing luciferase fusions with transcription factors or their protein stability domains can be considered as microbioreactors providing a way to directly monitor the stability of the luciferase labeled transcription factor or its domain in real-time. The quantitative analysis of the Neh2-luc reporter performance explains the multi-order shift in the apparent activation constant versus the "real" dissociation constant determined for a known Nrf2 displacement activator using fluorescent polarization homogeneous assay with recombinant Keap1 and labeled Nrf2 peptide. Apparently, the principal limitation for the performance of displacement activators is not their true dissociation constant, but the actual high micromolar concentrations of the endogenous Keap1, which results in lowering their biological effectiveness by many orders of magnitude. This conclusion derived from kinetic considerations has been confirmed by independent immunoblot assays of Keap1 and Nrf2 protein levels in cell lines as well as in murine liver and brain.

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From acetylcholinesterase inhibitors to multitarget ligands: a step forward in drug discovery for Alzheimer‘s disease

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Keywords: Alzheimer’s disease, cholinesterases, multitarget agents

Alzheimer’s disease (AD) is characterized by irreversible progressive loss of memory and other cognitive functions. AD treatments have focused on reducing cognitive decline with cholinesterase inhibitors as the treatment of first choice, and currently five approved pharmaceuticals are available for this purpose: galantamine, rivastigmine, donepezil, tacrine, and the NMDA receptor antagonist memantine. However, neurodegenerative diseases are multifactorial. The discovery of drug molecules capable of targeting multiple factors involved in AD pathogenesis would greatly facilitate improvements of therapeutic strategies.

One of the modern approaches for creating multitarget agents for AD treatment is polypharmacophore design — building hybrid molecules that are conjugates of two or more different pharmacophores linked together with spacers. Cholinesterase inhibitors are often used as one of the pharmacophores. We synthesized several series of hybrid structures combining certain pharmacophores essential for neurodegenerative disease treatment: tacrine, γ-carbolines, carbazoles, phenothiazines, and aminoadamantanes. Inhibitory activity of these conjugates against acetylcholinesterase (AChE), butyrylcholinesterase (BChE), and carboxylesterase (CaE) was studied. In addition, their ability to competitively displace propidium iodide from the peripheral anionic site of electric eel AChE was determined to assess their potential effect on AChE-induced aggregation of β-amyloid. Antioxidant properties were examined computationally with the DFT method and measured experimentally using ABTS and ORAC-FL assays. Binding modes of conjugates to AChE and BChE were studied using QM-assisted molecular docking. Results revealed conjugates that were selective inhibitors of BChE (tacrine/sulfamides) or that combined high potency and selectivity toward BChE with high radical-scavenging activity, e.g., γ-carbolines/carbazoles and tacrine/thiadiazoles. Conjugates of γ-carbolines with the phenothiazine derivative Methylene Blue and tacrine/thiadiazoles linked through an enamine-containing spacer demonstrated high potency against AChE and BChE combined with effective displacement of propidium from the peripheral anionic site of AChE. Additionally, the conjugates were extremely active in both antioxidant tests. All conjugates were poor CaE inhibitors, and were therefore expected to lack drug-drug interactions by this pathway. Good agreement was found between experimental and computational results. Lead compounds were identified for future optimization and development of new multi-target drugs against neurodegenerative diseases that combined cognition enhancement with neuroprotective potential.

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The role reactive oxygen species production in physiology and in mechanisms of neurodegeneration

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Neurodegenerative diseases are progressive, devastating and incurable, and are becoming increasingly prevalent in our aging populations. Second most common neurodegenerative disease - Parkinson's disease (PD) is a prevalent and progressive neurodegenerative disorder characterized loss of dopaminergic neurons in the substantia nigra pars compacta and the presence of Lewy bodies, which are intracellular inclusions of aggregated α-synuclein. A growing body of evidence points towards a key role of oxidative stress and mitochondria in PD pathogenesis. Oxygen is a highly active molecule and our organisms use it in their needs. Free radicals, which are constantly generated in enzymatic or non-enzymatic reactions, produce reactive oxygen species (ROS). In the process of evolution our cells learned how to use these ROS for their needs –in digestion, signalling and were able to protect themselves from oxidants in the cell via a very effective antioxidant system.

We have found that oligomeric alpha-synuclein do not produce ROS in mitochondria but induce non-enzymatic generation of free radicals. In familial form of PD, such a PINK1 mutation or deficiency, activation of NADPH oxidase induces oxidative stress responsible for deregulation of energy metabolism. Importantly, dopamine signalling activates monoamine oxidase (MAO) and induced further oxidation in familial form of Parkinson's disease. Production of free radicals from the different sources targeting mitochondrial enzymes and membranes and induced specific oxidative damage. Our findings further support importance of the oxidative damage in familial and sporadic form of Parkinson's Disease.

Design and development of antiplatelet agents.

Demina Olga

Analytical Application of Biocatalysis

Oligonucleotide functionalized nanoparticles for SERRS detection of short tumor-derived DNA fragments

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Keywords: ctDNA, cfDNA, TERT promoter mutations, SERRS

Annual increase of cancer cases determines the need for innovative biochemical methods for development of their early diagnosis and recurrence of oncological diseases. One of the modern trends is the development of non-invasive diagnostic methods based on the detection of tumor markers in biological fluids.

Analysis of literature data, genomic data for cell-free tumor DNA (ctDNA) based on targeted ultra-deep sequencing analysis with application of different amplicon size allowed to formulate a fundamental limitation of existing modern methods of detection of ctDNA for the development of non-invasive methods of diagnosis of tumor diseases. Such limitations base on predominant existence of very short fragments of tumor-derived cell-free DNA and were formulated as: 1) the need to optimize and minimize the pre-diagnostic treatment of body fluids, 2) the requirement to develop short amplicon-based or non-amplification methods for determining the sequence of tumor DNA, 3) the demand of high analytical sensitivity to tumor DNA against the background of the predominant presence of cell-free DNA (cfDNA). The found limitations substantiated the necessity of developing a fundamentally novel sensor system for molecular recognition of certain nucleic acid sequences.

As an example, for the development of the system, we select mutations in the promoter of the human telomerase catalytic subunit (*TERT*) gene. That mutations are characteristic of tumor DNA with potential practical application. Detection of mutations in the promoter of the human telomerase catalytic subunit gene in biological fluids may be the basis for non-invasive diagnosis of bladder cancer and some other types of tumor diseases.

The spectroscopy of surface enhanced (resonance) Raman scattering (SERRS) was selected as a possible method of detection due to it potentially can overlap all marked requests.

We investigated the possibilities of spectroscopy of surface enhanced (resonance) Raman scattering (SERRS) by structuring the surface of nanoparticles with DNA to detect certain sequences of nucleic acids and determining small quantities of nucleic acids. We established the optimal length between the Raman active labels and silver nanoparticle surface according to DNA size and could see the signal up to pM range of the Raman-label modified DNA.

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Association of molecular genetic markers of cell cycle control and DNA repair genes with progression free survival of ovarian cancer patients after platinum-based chemotherapy

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Keywords: ovarian cancer, platinum drugs, cell cycle control, DNA reparation, DNA methylation

The most important aim of the modern clinical oncology is the personalized treatment especially because of the high toxicity of chemotherapeutic drugs. The key drugs used in chemotherapy of ovarian cancer (OC) are platinum derivatives, which are both high efficacy and high toxicity. This makes relevant to search for sensitivity markers to this group of drugs.

The aim of this work was to study molecular genetic markers of DNA repair genes and the cell cycle control genes and their relationship with the progression-free survival time (PFS), which is a surrogate clinical marker of sensitivity of OC to platinum drugs. The study was included 31 patients with advanced OC (stage II-IV). The samples of tumor were selected from patients before the start of chemotherapy, during the primary cytoreductive surgery. All patients received standard chemotherapy using platinum drugs and paclitaxel after surgery. The polymorphic markers *Gln399Arg* of *XRCC1* gene, *Lys751Gln* of *ERCC2*, *Arg72Pro* of *TP53, T(-410)G* of *MDM2, Ser31Arg* of *CDKN1A*, and mutation *5382insC* of *BRCA1* were analyzed by PCR-RFLP and real-time PCR melting curves analysis as reference. Methylation of *BRCA1* and *TP53* promoter regions was studied in 19 pared probes of blood and cancer tissue using method of bisulfite conversion followed by methyl-specific real-time PCR. The results of analysis of markers were compared with the duration of PFS. A tendency towards a longer duration of PFS in the presence of *Gln* allele of *Gln399Arg XRCC1* ($p=0.07$) and a tendency towards a shorter duration of PFS in the presence of the *G* allele of the *T(-410)G MDM2* ($p=0.06$) was revealed. In the subgroup of patients with optimal cytoreductive sergery, a statistically significant decrease of the duration of PFS in the presence of the *Arg* allele of *Ser31Arg CDKN1A* was observed ($p=0.04$). A trend for shorter duration of PFS (median, M) in the presence of the *Pro* allele of the *Arg72Pro TP53* (M(*Pro*+)=11.8 months, M(*Pro*-)=17.0 months) and *Gln* of *Lys751Gln ERCC2* (M(*Gln*+)=14.1 and M(*Gln*-)=18.3 months) was found. Hypermethylation of *BRCA1* promoter in 3 samples of tissue was obtained. All these patients had no progression of disease and a trend to longer duration of PFS was obtained (18.0 compared to 15.1 months).

The obtained results suggest the advisability of further studying of these molecular genetic factors on a representative group of patients with OC.

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Enzyme-Linked Immunosorbent Assay (ELISA) With Photometric And Chemiluminescent Detection

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Keywords: Enzyme-Linked Immunosorbent Assay

The immunochemical methods based on the specific and high affinity antibodies are widely used for high-throughput screening (HTS) and monitoring of food contaminants. At first it is important for detection of poisons and toxic organic chemicals like antibiotics and mycotoxins. The Enzyme-Linked Immunosorbent Assay (ELISA) is one of widely used immunoassays. Moreover, ELISAs are modified to make its more simple, quick and cheap without any instruments or with using available device like smart phone. The chemiluminescent detection of ELISA could increase sensitivity of method, which using the same immunoreagents. Recent trend for development and application of new immunochemical methods for detection of food contaminants will be given. More details could be found in our recent publications.

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The use of vibrational spectroscopy for healthcare oriented biosensors

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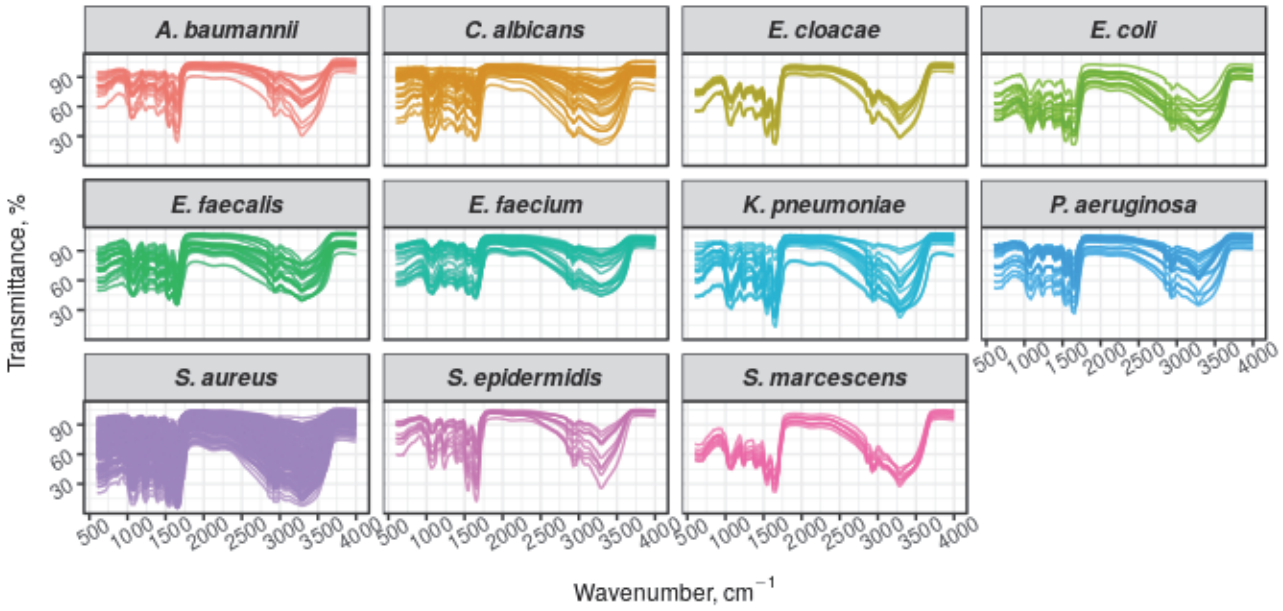
Keywords: SERS, FTIR, spectroscopy, multivariable data analysis, identification

The emergence of various new biological threats across the globe caused the worldwide increase in attention to matters of detection and identification of dangerous pathogens. Major efforts are directed towards the development of potential rapid and accurate techniques, that would allow detection and identification of pathogens at early stages in disease monitoring systems. It also arises from the massive spread of drug-resistant nosocomial strains and the emergence of centers for biohazard control. Vibrational spectroscopy methods, Fourier-transform infrared (FTIR) spectroscopy and Surface Enhanced Raman Spectroscopy (SERS) are a promising alternative to existing methods like mass spectroscopy. The aim of this work was to investigate the identification of microorganisms in pure cultures based on the analysis of vibrational spectoscopy. The algorithm is based on using FTIR and SERS spectroscopy and multivariate data analysis PCA-LDA approach: combination of Principal Component Analysis and Linear Discriminant Analysis.



Applying the HPLC-MS / MS method to identify and quantify oxidative post-translational modifications of proteins

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Keywords: (mass spectrometry, oxidative stress, fibrinogen, plasminogen, fibrin-stabilizing factor)

The adverse environmental-related factors as well as a number of the other, including various pathological conditions, can cause excessive production of reactive oxygen species (ROS) and reactive nitrogen species (RNS).

Proteins are the main targets for the action of ROS/RNS, which leads to the cleavage of peptide bonds, intra- and intermolecular cross-links, the formation of high-molecular aggregates or the oxidation of amino acid side chains. Oxidative post-translational modifications (PTM) of protein molecules are increasingly proposed to be used as markers of oxidative stress in various diseases. However, there is still no well-grounded theory that is capable in explaining the origin of specific oxidative sites, which may be markers of diseases.

Studies by the authors of this application have shown that not only the primary structure, but also the spatial organization of proteins play an important role in limiting the access of radicals to functionally crucial amino acid residues of a protein. According to this concept, plasma proteins have evolutionarily created various antioxidant areas (distinct subdomains, domains, individual polypeptide chains), the role of which, along with others, is to serve as ROS interceptors, thereby protecting the most vulnerable to oxidation amino acid residues, localized in other structural parts of the protein.

The goal of this project is to identify induced oxidative modifications of the human plasma proteins (for fibrinogen, plasminogen, plasma fibrin-stabilizing factor as example). We believe that these oxidative modifications, found in model systems with induced oxidation, can help one to obtain experimental evidence regarding the mechanism of the general structural adaptation of blood plasma proteins to the action of reactive oxygen species.

Information on the most susceptible to oxidation of amino acid residues in protein molecules will help with the targeted search for modifications in clinical studies of diseases characterized by the development of oxidative stress.

Authors also aim to elaborate new methodological approaches to identify the marker peptides for various diseases using the HPLC-MS/MS method. Developed software for quantitative assessment of PTM in samples.

The study was performed with the budgetary support of State assignment (subject no. 0084-2014-0001) and was supported by the RFBR № 18-04-01313_a, RSF № 19-14-00383. Mass spectrometry data were obtained with the support of the RSF № 16-14-00181.

Bioluminescent indication of the temperature stress on prokaryotic cells

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Keywords: bioluminescence, firefly luciferase, ATP, thermal stability, metabolic activity, prokaryotes

Monitoring of cell viability under the stress is one of the promising areas of research. The purpose of this work was to study the effect of temperature on the viability of *E.coli* expressing the mutant firefly luciferases, by measuring the bioluminescence intensity of the endogenous luciferase and the content of intracellular ATP.

The object of the study were *E. coli* BL21 DE3 Codon plus cells transformed with the pETL7 plasmid carrying the firefly luciferase *Luciola mingrelica* genes: wild-type (WT), thermostable (TS), and green (GTS) mutants. Luciferase was expressed in soluble and active form, and its enzymatic activity was measured by bioluminescence intensity of cells without their destruction on the luminometer (LUM 1, Russia) using a luciferin-based substrate mixture.

It was shown that in the temperature range of 42-60 ^{o}C there was a correlation between the enzyme activity of endogenous luciferase and the cell viability (CFU/ml, measured by culture method). Moreover, the activity of the enzyme isolated from the cells after their heating correlated with the activity of endogenous luciferase measured by bioluminescence intensity of intact cells. This indicated that the decrease in luciferase activity during heating occurs precisely due to the denaturation of the enzyme, and not due to a decrease in the concentration of the components necessary for the reaction of bioluminescence to take place.

The amount of intracellular ATP during the cell heating was determined by bioluminescent method for all *E. coli* cells producing luciferase mutants (WT, TS and GTS) and for non-transformed *E. coli* cells. For all samples, a sharp (3-5 fold) increase in ATP level was observed in the first 20 minutes of heating, followed by a gradual decrease in the signal. The rate of decline in ATP was significantly lower than the decrease in cell viability. It has been suggested that an increase in the rate of ATP synthesis at the initial stage of heating is associated with the activation of the protective functions of living cells under conditions of temperature stress, which requires serious energy expenditure.

Cytochrome P450 electrocatalytic systems for drug metabolism modeling, drug discovery and drug-drug interaction studies

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Keywords: cytochrome P450, electrocatalysis, screen printed electrode, drug-drug interaction, enzyme kinetics

Electrochemically driven cytochrome P450 (CYP) systems execute the dual function: substitute partner proteins and serve as a source of electrons for catalysis. Electrochemical CYP systems were applied to studying the catalytic activity, drug metabolism profiling, searching of substrate/inhibitor potential of newly synthesized chemicals, modulating of coupling efficiency, drug-drug interactions, the stoichiometry and the thermodynamics of the catalytic cycle. In electrochemical experiments, "*in electrode*" model system the catalytic current is an adequate expression of the enzymatic reaction. Electrocatalytic activity of mammalian cytochromes P450 2B4, 1A2, 3A4, 51b1 (sterol-14α-demethylase *Mycobacterium tuberculosis* CYP51b1), 11A1 (P450scc), 2C9, 2D6, 17A1 were studied using different types of modified electrodes. The drug-drug interactions for mammalian CYP3A4, CYP2C9 and CYP2D6 were studied for diclofenac, erythromycin, testosterone, metabolic antioxidant preparations such as ethoxidol (2-ethyl-6-methyl-3-hydroxypyridine malate), mexidol (2-ethyl-6-methyl-3-hydroxypyridine succinate), cytochrome *c*, L-carnitine, , taurine, antihypoxant mildronate. Drug electroxidation was used for estimation of drug determination. These findings provide data for future clinical risk prediction studies – especially for those devoted to the interaction of drugs with antioxidants and antihypoxants. Electrochemically driven CYP reactions may have practically relevant providing a useful tool for drug assay studies.

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Polyelectrolyte microcapsules with enzymes for use in analytical systems

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Keywords: polyelectrolyte microcapsules, electrochemical biosensors, glucose oxidase, urease, nanoparticles

In recent years biosensors and biofuel elements have become widespread along with the traditional methods of analysis in clinical diagnosis and analysis of food quality. One of the ways to preserve the activity of biocatalyst when developing such bioanalytical systems is its immobilization in polyelectrolyte microcapsules. Such capsules protect the biocatalyst from the aggressive impact of the environment and do not prevent the penetration of substrate into the capsule. The inclusion of the enzyme inside the microcapsule allows you to keep the activity of the enzyme in the biosensor at a high level for several months. Electrochemical sensors are one of the methods for determining the activity of encapsulated enzymes. For example, the activity of glucose oxidase can be determined by the amount of hydrogen peroxide generating as a result of glucose oxidation. We used graphite electrodes modified by Prussian blue, which is a catalyst for the electrochemical reduction of hydrogen peroxide. Urease activity was determined using a pH-sensitive field effect transistor by changing the pH as a result of the decomposition of urea under the action of an enzyme. We studied the characteristics of the obtained biosensors for the determination of glucose and urea (the dependences of biosensor signals on pH and molarity of the buffer solution, on NaCl concentration; determination limits; the operational and long-term stabilities). Immobilization of microcapsules with an enzyme on the electrode surface can be performed by various methods. In our research, we were based on the following principles. The top layer of polyelectrolyte covering the capsule carries a negative charge and adheres well to the surface of the graphite electrode due to the Coulomb interaction. In the case of a pH-sensitive field-effect transistor with tantalum pentoxide surface, we can additionally introduce paramagnetic particles of Fe_3O_4 into the capsule to form the bioreceptor using a permanent magnetic field directly on the transistor gate.

Polyelectrolyte capsules were additionally modified with multi-walled carbon nanotubes to increase the sensitivity of the developed biosensors.

The developed biosensors were used for blood, milk and commercial juices analysis. High correlation of data with standard spectrophotometric methods of analysis was shown. Thus, the method of polyelectrolyte encapsulation of enzymes is promising in the development of biosensors and microbial fuel cells and will find application in biotechnology, medicine, power engineering and robotics.

Acknowledgement. The development of biosensors for the detection of glucose was supported by a Russian Science Foundation grant №18-29-23042. The study of the effect of electrode modification by nanoparticles was supported by a Russian Science Foundation grant №18-577802.

New approach to identification of microorganisms with Fourier-transformed infra-red spectroscopy

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Fast identification of potential pathogenic microorganisms remains a challenging task of the modern biomedicine and namely clinical diagnosis. Mass-spectroscopy approach is the main-stream in this field. This is a time-efficient method with an extremely high resolution. Ubiquitous use of Biotyper by Bruker Daltonics and Vitek MS by BioMerieux provides extensive databases including thousands of relevant microorganisms. However, mass-spectrometry remains an expensive method unaffordable for routine use in infectious diseases departments of common hospitals. Fourier-transformed infra-red (FTIR) spectrometry is based on inexpensive instruments and does not require disposables. Feasibility of this principle is proved by commercial availability of IR Biotyper instrument by Bruker. However, FTIR remains just a method of choice for identification of microorganisms used only in several laboratories worldwide.

Recently we proposed a novel approach to composing FTIR data bases for common microorganisms and algorithms of their use for identification of novel isolates. It is based on linear discriminant analysis and includes an original procedure of etalon spectra collection. In contrast to other groups, we cultivated collection bacterial isolates under a series of drastically different conditions (medium composition and time of cultivation are varied in a maximally available extent). The obtained spectra were analyzed by the soft-ware which identified components of the spectra attributed to the microorganisms species under any conditions. The obtained data are stored as an array of variables allowing a fast and unambiguous analysis of new isolates.

This approach was formerly tested towards highly prevalent agents causing purulent and nosocomial infections: *Staphylococcus aureus, Enterococcus faecalis, Enterococcus faecium, Klebsiella pneumoniae, Escherichia coli, Serratia marcescens, Enterobacter cloacae, Acinetobacter baumannii, Pseudomonas aeruginosa* and *Candida albicans*. The algorithm exhibited a high robustness however in the course of trials it failed to distinguish methicillin-resistant *S. aureus* strains from methicillin-resistant ones. Moreover, common human commensals - non-pathogenic representatives of genus *Staphylococcus, e.g. Staphylococcus epidermidis, Staphylococcus haemolyticus* and *Staphylococcus hominis* are poorly discriminated from *S. aureus* isolates.

We supposed that the experimentally found inefficiency of the algorithm was caused by lack of non-pathogenic Staphylococci in the panel of etalon spectra rather than principal failures of the method. In order to check this hypothesis, we collected a panel of non-pathogenic Staphylococci: *Staphylococcus capitis* - 9 isolates, *S. epidermidis* - 126 isolates, *S. haemolyticus* - 44 isolates, *S. hominis* - 36 isolates, *Staphylococcus sciuri* - 12 isolates, *Staphylococcus simulans* - 14 isolates, *Staphylococcus warneri* – 12 isolates. All these isolates were subjected to the routine procedure reported formerly and a perfect identification of *S. aureus* was achieved in blind trials including 188 isolates.

The described experiment gives evidence that FTIR can be suggested as a universal approach to identification of microorganisms in clinical diagnosis and initial detection of pathogens in the out-of-door space. It is highly competitive to MALDI-TOF in terms of costs and resolution. However, database collection must be completed before this method becomes commonly available for a routine application.

Development of a diagnostic approach based on the detection and quantification of sites of post-translational modifications using the HPLC-MS / MS method in the protein components of blood plasma

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Keywords: (mass spectrometry, oxidative stress, fibrinogen, plasminogen)

Nowadays, studying of oxidation of proteins, including plasma proteins, is one of the most upcoming scientific area in free radical biology and medical science. Proteins as a vulnerable target to reactive oxygen species are involved in oxidative modifications which proceed with structural and chemical damages. Oxidative protein modification might cause a significant loss of biochemical functions of protein.

With aid of the results obtained in the framework of the study, by using the HPLC-MS/MS method it is possible to create fundamentally new diagnostic approaches for clinical and laboratory practice based on the detection and quantification of oxidative sites resulting from the development of oxidative stress in various pathologies.

In the framework of the study first stage, the following methods based on immunoprecipitation on magnetic particles (for plasminogen and fibrinogen) were developed and adapted: 1) enzyme immunoassay on magnetic particles to determine the amount of precipitated proteins; 2) trypsinolysis on magnetic particles with the exception of the elution stage for further analysis by high performance liquid chromatography and tandem mass spectrometry (HPLC-MS/MS). Development of trypsinolysis techniques on magnetic beads with the exception of the elution stage for further analysis (HPLC-MS / MS) allowed: 1) to simplify procedures when working with a large number of samples, 2) to reduce the total analysis time, 3) to avoid the appearance of oxidative modifications not related to pathological conditions or induced by oxidation, but resulting from prolonged sample preparation or using aggressive elution conditions, those thus impeding the result analysis.

The development of such research will lead to a greater understanding of the role of oxidative modifications of proteins in normal and pathological conditions.

The study was performed with the budgetary support of State assignment (subject no. 0084-2014-0001) and was supported by the RFBR № 18-04-01313_a, RSF № 19-14-00383. Mass spectrometry data were obtained with the support of the RSF № 16-14-00181.

Biopolymer nanofibrous materials for tissue engineering and medicine

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Keywords: (electrospinning, polyhydroxybutyrate, release of therapeutic agent, fibrous materials)

Nowadays, one of the topical issues in the field of health systems is the development and application of innovative nanoscale materials with special properties. It is important to stress that such materials should have high operational characteristics, also they must have all the properties, which are necessary for medical products, and they must satisfy all the modern requirements of rapid biodegradation after use. Among the promising objects, which comply with all these requirements, are nonwoven materials with various additives based on biopolymers obtained by electrospinning method. Nanocomposites for medicine and tissue engineering could be made based on such materials.

The paper considers and analyses the factors that can have an effect on the kinetics of the release of drugs from fibers based on biopolymer – polyhydroxybutyrate (PHB). The results were used as the basis for the design and manufacturing of "smart" therapeutic drug delivery systems, elements applicable in tissue engineering, implants and other products, which are in contact with the liquid media of the human body. In addition, the mechanism of release of drugs from the matrix based on PHB fibers was established in the following work. The characteristics that can define the morphology of matrices based on non-woven and ultra-thin nanoscale biopolymer fibers were studied and classified, among which the diameter of the fibers, the nature of their mutual were stacking, the pore size, the presence of inclusions and microdefects, the degree and quality of attachment with a polymer film carrier.

The paper experimentally confirmed the effectiveness of ultra-thin fibers, the size of which is comparable to the body fibrils, as matrix elements. The studied characteristics have a significant effect on the kinetics of medicinal substance (endoxan) release in buffer solution, which imitates a living system that is similar to the course of bioresorbable biopolymer structures in an organism.

The samples of fibers and nonwovens were obtained by electrospinning method from the solution, the morphology and structure of the material were evaluated by the scanning and optical microscopy, biodegradation of materials was studied by UV spectrometry, the study of experimental samples under *in vitro* conditions was carried out using human colon adenocarcinoma cells and cytotoxic test was carried out.

Nanomaterials and Nanotechnologies

Possible modes of influence, state and prospects for the application of variable magnetic fields as a means of controlling biochemical systems

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Keywords: alternating magnetic field, magnetobiology

An alternating magnetic field (AMF) can affect the state of biochemical objects of various scales, degrees of complexity and hierarchy (from single bioactive macromolecules to organisms), physico-chemical processes in them and functions in many ways. Such a multimodality means that under the action of the AMF, a variety of very diverse physical mechanisms can be activated, often operating simultaneously, in parallel, sequentially or alternately. This makes the interpretation of many well-known and objectively existing magnetobiological phenomena, the prediction of new and their practical use is much more complex than that arising under the action of heat, mechanical stress, ionizing radiation and other thermodynamic environmental factors.

The lecture provides a classification of variable magnetic fields in terms of the effectiveness of various possible channels of action on biophysical objects and systems. Possible molecular targets and the nature of the primary acts of action of the AMF, the "*kT* problem", spin-dependent magnetically sensitive reactions, etc. are discussed.

The second part of the lecture is devoted to the consideration of a physically more specific situation when magnetic / superparamagnetic nanoparticles (MNPs) are preliminarily introduced into a biophysical system of one nature or another with known magnetic, geometrical and physicochemical characteristics. Then, the system is acted on by the controlling AMFs or their various combinations in order to induce the intended (planned) response in it. The main and radical difference between this situation and the conditions from the previous one (without the presence of MNPs in the system) is that the obvious and most likely targets of the action of the AMF are not individual atoms, macromolecules or biomolecular structures, but MNPs with a magnetic moment many thousands of times greater than that may be in a biomolecular structure. This leads to a huge concentration of energy given by the AMF to the macromolecules in one way or another interacting with MNPs. However, in this case there are several ways and even more possible types of responses of the biomolecular system to the action of the AMF. The thermal mode of the influence of radiofrequency AMF (magnetic hyperthermia) and its main features, advantages and disadvantages in usually used AMF frequencies 200-800 kHz, as well as non-thermal mode, called nano-magneto-mechanical actuation (NMMA) and implemented in non-heating AMF with a frequency of <1 kHz, will be briefly discussed. The latter has been actively developing in recent years and has proven its versatility, safety and availability in a wide variety of biomedical tasks (targeted drug delivery, controlled release from nanosized carriers, drug-free induction of apoptosis of malignant tumor cells through mechanotransduction, but not a heating, etc.). In this capacity, NMMA can be considered as the biomedical section of the actively developing in recent years the new scientific direction of "Straintronics", implying the control of the properties of objects and systems for various purposes through their precise deformation at the nanoscale.

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Nanotechnology strategies using oxime-loaded lipid nanoparticles for rat brain protection against paraoxon

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Keywords: Drug delivery systems, Oxime, Blood-brain barrier, Acetylcholinesterase, Organophosphates

Nowadays, the main strategies for brain protection against organophosphate pesticide are: i) synthesis of new reactivators of acetylcholinesterase (AChE) that effectively cross the blood-brain barrier (BBB) and act in the central nervous system (CNS) [1,2] ii) creation of delivery systems for reactivators of AChE (quaternary oximes). Oximes-loaded nanoparticles capable of penetrating the BBB can be injected in the bloodstream or alternatively can reach directly the CNS through the nose-brain pathway [3,4].

Our research group focused on the encapsulation of marketed quaternary oximes (2-PAM, HI-6 and obidoxime) using solid lipid nanoparticles (SLNs) and cationic liposomes [5,6]. SLNs are biocompatible, biodegradable and have a very low toxicity, thereby fulfilling the requirements of preclinical safety [7]. "Two-in-one" approach using SLNs for packaging two oximes in single carriers for brain protection against organophosphate pesticide has been developed. 2-PAM in association with a novel a poorly water soluble 6-(5-(6,7-dimethoxy-3,4-dihydroisoquinolin-2(1H)-yl)pentyl)-3-hydroxypicolinaldehyde oxime (3-HPA), were loaded into SLNs to provide a sequential time-release profile with different half-lifes for both oximes [8]. To increase the therapeutic time window of both oximes, two- compartment SLNs were designed. Oximes in SLNs displayed longer circulation time in the bloodstream compared to free 3-HPA and free 2-PAM. Oxime-loaded SLNs were suitable for intravenous administration. Paraoxon-injected rats ($0.8\times LD_{50}$) were treated with 5mg/kg of 3-HPA-loaded SLNs and 2-PAM+3-HPA-loaded SLNs. For combination post-exposure therapy with these two encapsulated oximes, a time-dependent additivity of oxime effect with increased AChE reactivation up to 35% was observed.

In addition, new mixed cationic liposomes based on L-α-phosphatidylcholine and cationic lipid were administered via the intranasal route. This last approach provides evidence that reactivation of central paraxonon-inhibited AChEs can be achieved by a non-invasive approach. Further improvements of treatment efficiency expected by modifying the surface of lipid nanoparticles with enhancers, improvers of BBB permeability and receptor-specific ligands.

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Lipobeads: Mechanisms for Controlled Release of Encapsulated Anticancer Drugs

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Keywords: (nanoparticles, lipobeads, anticancer drugs, drug delivery, mechanisms)

The ultimate goal is to develop anticancer chemotherapy with superior tumor response and minimum side-effects even at a greater drug loading concentration. Lipobeads (50–180 nm) are good candidates for bio-signaling regulated drug delivery [Kazakov S. Liposome-nanogel structures for future pharmaceutical applications: An updated review. *Current Pharmaceutical Design* 2016, 22(10), 1391-1413]. Conceptually, lipobeads bring about attractive properties from both liposomal and polymeric systems in one construct. From the liposomal systems, the most important benefits they retain are well developed methods for preparation, efficient encapsulation of poison molecules, and biocompatibility of the lipid bilayer. On the other hand, the hydrogel core provides mechanical stability to the lipid bilayer and environmental responsiveness to the overall structure. Three mechanisms of drug release from lipobeads, depending on the responses of the hydrogel core (such as swelling, contraction, and degradation), can be designated. **"Sponge-like" mechanism:** the hydrogel core initially is in a swollen state. When the environment changes (temperature, pH, etc.), the polymer network shrinks, so that the hydrogel core releases the loaded drug like a squeezed sponge into the space between gel and lipid membrane, and the drug slowly (hours) diffuses through the membrane into the external environment. **"Poration" mechanism:** the hydrogel core initially is in a shrunken state and drug molecules are trapped more tightly within the polymer network, so that their release can be even more suppressed in comparison with conventional liposomes. When the environment changes, the polymer network swells so much that the volume of hydrogel core becomes greater than the space provided by the closed lipid bilayer; the "growing" hydrogel core causes disruption of the lipid bilayer, and pore formation results in the drug release through the pores. This mechanism provides a way of drastic increase in the rate of drug release (minutes) in response to stimuli. **"Burst" mechanism:** "exploding" lipobeads could be fabricated, if their hydrogel cores are made of a biodegradable polymer network. If the interchain cross-links can be cleaved by hydrolysis or redox reaction, the swelling pressure inside the lipobeads increases, so that, at some instant, the internal pressure becomes sufficient to break the membrane and encapsulated drug falls out of the lipobeads with the maximal release rate (seconds). We synthesized a series of bulk fluorescent hydrogels and microgels with different responsiveness to external stimuli. "Thermophobic" polymer networks, which collapse upon heating and are suitable for the "sponge-like" mechanism, were prepared using *N*-isopropylacrylamide and *N*,*N*-methylenebisacrylamide (MBA) as monomer and cross-linker, respectively. For testing lipobeads with the "poration" mechanism, "thermophilic" hydrogels, which swell upon heating, were prepared by co-polymerization of acrylamide and acrylonitrile in the presence of MBA as the cross-linker. The hydrogels degradable in highly reducing environments (such as those found in cancer cells) were synthesized using *N,N'*-bis(acryloyl)cystamine as a cross-linker with disulfide bridges. Technological aspects of lipobead preparation by polymerization within lipid vesicles and nanogel/liposome mixing are discussed in this presentation. Giant lipobeads with a "thermophobic" or "thermophilic" core and with or without redox-degradability were prepared by mixing corresponding microgels with liposomes. Their morphology and structure were studied by confocal and scanning electron microscopy to justify spontaneous formation of a lipid bilayer on the surface of microgels, evidencing energetically favorable structural organization of the hydrogel/lipid bilayer assembly. In addition to the aforementioned schemes of drug release, the bi-compartmental structure of lipobeads could provide a number of novel and unique options such as consecutive multistep triggering and combined drug delivery systems.

Inclusion of drugs into nano- and microparticles for use in ophthalmology

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Keywords: inhibitors of angiotensin-converting enzyme, superoxide dismutase, polymeric nanoparticles, calcium-phosphate microparticles

It is known that only about 5% of the drug applied in the form of solution instillations can reach intraocular tissues. This problem can be solved by the use of nano- and microcarriers capable to improve the efficacy of drug delivery into the eye by overcoming corneal/sclera diffusion barrier. In particular, it is very important for the treatment of eye diseases accompanying by oxidative stress, as the transparency of the cornea and lens, as well as the functioning of photoreceptor apparatus, relies on their highly ordered structures, and excessive tissue damage by reactive oxygen species will compromise visual function. Here we describe a therapeutic potential of antioxidant enzyme, superoxide dismutase I (SOD), incorporated into polymeric nanoparticles and inorganic calcium-phosphate (CaPh) microparticles covered by cellobiose, in the treatment of inflammatory eye diseases accompanying by oxidative stress. The study was carried out in the experimental models of inflammation in intraocular (immunogenic uveitis) and outer (corneal alkali burn) structures of the rabbit eye. It was shown that SOD within particles was statistically more effective in therapy of uveitis than SOD in solution in such inflammatory manifestations as corneal and iris edema, hyperemia of conjunctiva and eyelid, lens opacity, and the protein content in aqueous humor. Moreover, the amount of fibrin clots in the anterior chamber of the eye, leading to the postuveal glaucoma, was significantly lower in rabbits treated with SOD within particles than in ones receiving instillations of SOD solution. SOD in CaPh-particles was also shown to be more effective in the treatment of alkali eye burn. We registered its higher anti-inflammatory and anti-ulcer activity, higher transparency of cornea after the wound healing, decrease of ulceration area and depth. Thus, *in vivo* experiments demonstrated the therapeutic advantage of SOD incorporated in nano- and microparticles of different nature over SOD in solution.

As a more challenging approach we investigate incorporation of low molecular weight compounds, beta-adrenoblocker timolol and inhibitors of angiotensin-converting enzyme (ACEI) into CaPh-particles coated by cellobiose or chitosan. The inclusion of both timolol and ACEI into CaPh-particles proved to be very effective, as this resulted in substantial enhancement and prolongation of hypotensive effect on the intraocular pressure compared to that by the compounds in solution. Thus, inorganic matrices could be quite effective as carriers of small molecules for use in ophtalmology.

Functionalized lipid drug delivery containers

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Keywords: drug delivery, liposomes, fluoroquinolones

Development of drug delivery systems is one of the most important tasks of current state-of-art in biopharmaceutics. New antibiotics able to fight infectious usually possess limited bioavailability and a number of side effects. One of the perspective approach to overcome these drawbacks is to encapsulate active molecule into liposomes, however further functionalization of liposomal surface is required.

Chitosan coating was previously used in our lab as the beneficial strategy of liposomal properties managing via complex formation. It is well-known, that alveolar macrophages, typical host for *Mycobacterium Tuberculosis*, overexpress mannose-binding receptors. Thus, current research is dedicated to develop functionalized liposomal form of fluoroquinolones with active targeting via mannose moiety and to study chitosan-mannose conjugate as a functionalizing agent for liposomal form of fluoroquinolones including synthesis, properties, influence on the membrane.

It was possible to achieve a reduction in the synthesis time of the chitosan-mannose conjugate up to 12 hours compared with a literature time of 48 hours. The concentration of the modifying agent and the reaction time were varied in order to detect the dependence of the degree of chitosan modification on the synthesis conditions. An original method was proposed for determining the degree of modification of chitosan derivatives based on FTIR spectroscopy; titration of free amino groups by TNBS was used as a control method. It was firstly demonstrated, that the degree of modification substantially depends on the molecular weight of the initial chitosan and the molar excess of mannose. Thus, for chitosan 5kDa, regardless of the molar excess of mannose (5-25), the degree of modification remains at 35-38%, while for chitosan 90 kDa, the same excess of mannose leads to the production of various products with a characteristic pattern. This effect seems to be associated with a large number of available free amino groups of chitosan 5kDa, which contributes to easier modification of the surface of the biopolymer.

To obtain liposomes loaded with moxifloxacin (Mox) and levofloxacin (Lv) by the method of passive loading the thin lipid film was dispersed with buffer solution containing the drug. To obtain LMox and LLv by the method of active loading an ammonium sulfate gradient was used. To determine the mechanism of interaction of antibiotics with the liposomal membrane, a complex of spectral methods was used, namely Fourier ATR-FTIR, UV spectroscopy, DLS and NTA. The loading efficiency was greater for moxifloxacin compared with levofloxacin, apparently due to its greater lipophilicity, while the use of ammonium sulfate gradient allowed the loading efficiency to be increased by 10–20%, which is a new result.

Complex formation leads to growth of hydrodynamic radius of vesicles and neutralization of ζ-potential. ATR-FTIR spectroscopy data reveals main binding sites: phosphate and carbonyl groups of lipids and allows investigate drug location in membrane. Changes in membrane depends on membrane composition and polymer composition. Complex formation with chitosan derivatives, as we found, modulates drug release: in 0,02 M PBS solution pH 7,4 release is significantly prolonged and in 0,02 M sodium – acetate buffer solution pH 5,5 release is accelerated.

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Polyacrylate of gold is a candidate chemotherapeutic agent for curing melanoma

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Polyacrylate of gold (PG) was formerly described as an agent able to contain a tumor growth caused by grafted carcinomas Akatol, Lewis and Ca-755 in mice. We modified technology of PG synthesis and succeeded improving conversion of Au^{3+} ions to the final product from 15.6% to 99.8%. The new method requires a polyacrylic acid with an exactly determined molecular weight 140 kDa. Before reaction, it must be thoroughly desalted with a dialysis and must be introduced to a reaction with tetrachloraurate (molar ratio 1:5 of $HAuCl_4$ to a polyacrylic acid monomer) at a high concentration 10%w. The final product must be subjected to a repetitive dialysis for removal of low molecular mass reaction products (*e.g.* HCl). PG manufactured by this method exhibited a high stability at storage at +4°C in a liquid form (concentrated solution) or as a lyophilized powder. The lyophilized powder is water soluble at concentration >29.3 mg/ml, it contains ~21 μmol/mg Au^{3+}.

Testing cytotoxic activity of PG manufactured by different methods against MCF-7 cell line (human breast cancer) gave evidence that it depended on concentration of Au^{3+} only (IC_{50} always was 1.7 mM Au^{3+}). Concentration of polyacrylic acid did not affect the cytotoxicity.

PG synthesized following the novel method was tested as a cytotoxic agent for cultivable human melanoma lines MEL-P, MEL-IL, MEL-Z, MEL-Wo, MEL-MTP, Mel-Me and MEL-IBR provided by Blokhin cancer scientific center and B-16 murine melanoma line (available from ATCC). Surprisingly, the cell lines exhibited different sensitivity to PG. MEL-P, MEL-IL, MEL-Z and MEL-Wo were highly sensitive (IC_{50} varied in range 50-120 μM), MEL-MTP, Mel-Me and MEL-IBR had a moderate stability (IC_{50} 200-250 μM) whereas B-16 was very resistant to PG (IC_{50} = 450 μM).

As far as the tested melanoma lines were formerly characterized by expression profile by using hybridization on high-density chips, data about expression level of a broad range of lymphocyte-specific superficial antigens was available for them. These data were tested for correlation with sensitivity of the lines to PG and following correlations were found. Expression level of CD80 (a superficial marker of mature B-lymphocytes) demonstrated a direct correlation with IC_{50} value. Therefore, elevated expression of this marker raises cell resistance to PG. In contrast, elevation of CD3, a universal marker of lymphocytes, correlated with decreased IC_{50}, therefore high level of this protein makes the cells sensitive to cytotoxic action of PG. Similar trend is found in HLA-DR expression (component of human MHC-II). Taken together, these data allow a speculation that superficial protein markers may contribute to transportation of PG or Au^{3+} or protect the ion from a reducing with non-specific target molecules.

Searching for specific targets of PG in tumor cells led us to an observation that PG is able to an efficient inactivation of fluorescent dyes *e.g.* propidium iodide, bis-bensimidazole (hoechst 33342), Cyber-green and Rhodamine 123. The effect is found in near stoichiometric ratios and leads to bleaching of fluorescent dyes used for staining different cell structures at confocal microscopy. In vitro studies demonstrate the these reaction lead to oxidation of the fluorescent dyes. The reaction goes fast at μM range of concentrations and is not prevented with a high excess of proteins, lipids, DNA and other biomolecules found in the whole cells. Therefore, one should speculate that these type reactions mimic processes leading to the cell killing by PG.

As a conclusion, we should report that PG may be suggested as a candidate agent for therapy of melanomas human. So far, standard cytotoxic preparations are not used for treatment of melanomas due to their low specific efficacy even at high dosages leading to an unacceptable general toxicity for the patient. We compared ration of the specific cytotoxicity to melanoma B-16 and acute toxicity of PG and a reference commercial preparation Cysplatin in mice. Cysplatin was chosen as a broadly used commercial cytostatic agent relatively close to PG by its chemical composition (both medicines contains ions of noble metal). Average specific activity (IC_{50}) for PG and Cysplatin was 115 and 36 μM, acute toxicity in mice (LD_{50}) was 670 and 39,65 μmol/kg. The ratio of the specific activity to the acute toxicity was 0,17 and 0,91. Therefore, selectivity of PG to the target melanoma cells is 5,3 times higher than in Cysplatin. These calculations allow suggestion about a high potential of PG as a candidate medicine for treatment of temporary blockade of melanoma focuses in human.

Biotechnological experiments at orbital space stations and perspective use of nanotechnology for commercial application

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Keywords: space biology, microgravity, plant

Biological experiments in a field of space biology have been started before the first satellite flight. These experiments were devoted to an estimation of space radiation factors on living organisms and carried out in mountains. The systematic biological experiments in space have been started in 1971 with orbital station Salyut. In total more than 1000 experiments have been installed in space flights: fundamental investigations (panspermia theory, gravity biology, complex factors of space environment on biological objects) and applications focused on future biological life support systems. The investigations were directed to some tasks: influence of complex factors of space flight on living organisms at different stages of the evolution scale; investigations of proteins and DNA, cell, tissue, organism and assembled organisms under space flight factors with separation of individual factors, for example, microgravity and space radiation. The aim was to understand the organism reactions on different levels, to get complete ontogenesis cycle in space flight and to find adaption ability of organisms to extreme factors of the space flight. In course of investigations, the unique experimental equipment for orbital biological experiments has been designed; new methods for organism protection against the negative factors of space flight were found; developed new biotechnological products and processes; developed recommendations for space station interior with biological objects for psychological comfort of crew. The results showed a possibility and ways to include different organisms into biotechnological life support systems for future space stations and interplanet spaceships.

Polypeptide nanoparticles as potential drug delivery systems

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Keywords: polypeptides, self-assembled nanoparticles, drug delivery systems, encapsulation

Amphiphilic copolymers capable of self-assembly in aqueous media represent the convenient and useful tool to create nanoparticles with required morphology, size, stability, etc. The feasibility of variation of particle's properties favors to the application of amphiphilic copolymers as delivery and detection systems. In aqueous media, the amphiphilic copolymers are self-assembled into the spherical and cylindrical micelles, polymersomes and non-ordered nanospheres. Contrary to the micelles that have hydrophobic core and hydrophilic envelope, polymersomes are characterized with hydrophilic inner core and outer particle surface and hydrophobic polymer membrane. Comparatively to other polymers, polypeptides are related to the class of biocompatible and biodegradable polymers that are very attractive for the different fields of bioapplication.

In this work, the different amphiphilic random and block-copolymers were synthesized. Generally, the copolymers obtained can be divided into three groups: (1) the copolymers of L-amino acids, (2) copolymers of L- and D-amino acids, and hybrid copolymers based on synthetic glycopolymers and L-amino acids. Depending on the polymer composition the self-assembly in the aqueous media provided the formation of polymer particles with the size ranging from 90 to 700 nm. The self-assembled polymer systems demonstrated the different stability to biodegradation that allows for the management of drug release rate. All developed systems were carefully tested for the cytotoxicity with the use of different cell lines. Moreover, the ability to penetrate inside the cells because of non-specific endocytosis was established. To monitor the cell uptake of nanoparticles, the fluorescent nanoparticles were prepared and applied for the testing. Additionally, the phagocytosis of the developed polypeptide and hybrid polymer particles was studied in the experiments with macrophages.

The self-assembled polypeptide and hybrid copolymers were suitable for encapsulation of wide range of drugs and biological compounds: charged (peptides, siRNA, DNA), hydrophobic (glucocorticosteroids and taxanes) and amphiphilic (cytostatic drugs and dyes). The features of encapsulation of different compounds, encapsulation efficiency and maximal drug loading into polymer particles were established. The conditions of drug release were studied and the biological efficacy of encapsulated compounds was proved in the cell culture experiments.

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Glycosaminoglycans based nanogels for ocular drug delivery

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Keywords: nanogels, pDNA, siRNA, intracellular delivery, enzyme-sensitive

Intracellular delivery of biopharmaceutics, such as proteins and nucleic acids (NA), represents an actual direction of research that can fundamentally change the existing therapy of a number of diseases, such as monogenic diseases, metabolic disorders, oncological diseases etc. Of particular interest is the treatment of ocular disorders, such as neovascular glaucoma (NVG) and age-related macular degeneration (AMD). NVG is one of the most refractory types of glaucoma caused by high ischemic retinal disorders, resulting in severe visual loss. AMD is the leading cause of blindness in the Western world for those patients aged 50 years or older. Neovascular AMD, a subtype characterized by the growth of new, pathologic blood vessels, and results in most of the cases of severe and rapid vision loss.

For the treatment of neovascularization the inhibition of vascular endothelial growth factor (VEGF) or is gene knockdown is needed. Currently, anti-VEGF monoclonal antibody – bevacizumab (Avastin®) – is commonly used for treatment of described situations. However, this drug is not free from side effects and better drugs, as well as drug delivery systems are on demand. The novel "genetic drugs", such as pDNA and siRNA could be used for the *in situ* synthesis of anti-VEGF inhibitor or VEGF gene knockdown, respectively. Obviously, that in their free form such drugs could not reach their intracellular targets and will possess unsatisfactory pharmaceutical properties.

Within the presented study, we have developed nanogels for intracellular delivery and pH- or enzyme-sensitive release of pDNA or siRNA. To obtain such systems the polycation (chitosan, polylysine and polyarginine) was used to condensate the nucleic acid (NA). The positive charge of obtained systems could prevent their diffusion in hyaluronic acid rich vireous liquid. In order to compensate the positive charge of the polyplex the glycosaminoglycans (GAGs: heparin and chondroitin sulfate) were applied. The ionic gelation of polycation – GAG systems at special conditions (concentration, charges relation, ultrasound) results in the formation of nanogel. Such nanogels are very soft and swellable systems with size from 30 to 80 nm, according to TEM and DLS measurements. They allow encapsulation of pDNA with 80 % and siRNA with 100 % efficacy, correspondingly. The release of NA from such interpolyelectrolyte systems is pH dependent. Nevertheless, in order to stabilize nanogels and provide the possibility for intracellular trigged release the pH-sensitive ketal linker and cathepsin B-sensitive linker were used to cross-link the nanogels. The triggered release of NA from obtained nanogels was studied, as well as their cytotoxicity, cell penetration, transfection and gene knockdown ability. The possibility to stabilize the encapsulated NA from extracellular enzymes action by encapsulation in nanogel was studied and proved. Also, the enzymatic reactions were used to study the structure and stability of nanogels themselves.

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Magnetic liposome responsive to super-low frequency alternating magnetic field

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Keywords: magnetic liposomes, lipid composition, drug release, super-low frequency AC MF

Magnetic liposomes are developed for targeted delivery and "on demand" release of drugs. The project investigates magnetic liposomes of various compositions containing hydrophobized iron oxide magnetic nanoparticles (MNPs) in lipid membranes. Such liposomes are shown to release the entrapped dye once modulated by low frequency alternating current magnetic field (AC MF). Their exposure to the AC MF (50 Hz, 15 to 110 kA/m) results in the clustering of the MNPs in the membranes, loss of liposomal structure and disruption of the lipid packaging as determined by the transmission electron microscopy (TEM) and attenuated total reflection Fourier transfer infrared (ATR-FTIR) spectroscopy. The field induced dye release depends on the lipid composition. PEGylation of liposomes increases their stability and slightly decreases the release. Addition of cholesterol (Chol) greatly diminishes the dye release from the saturated lipid 1,2-Distearoyl-sn-glycero-3-phosphocholine (DSPC) based liposomes. Replacement of the DSPC for unsaturated lipid egg L-α-phosphatidylcholine (eggPC) also decreased the dye release. The dye release depends on the temperature as well as the strength, but not the frequency of the field (50 Hz vs 1000 Hz). The use of DC fields of similar strength does not release the solutes from the magnetic liposomes. We posit that the oscillating motion of MNPs in AC MF disrupts or ruptures the gel phase membranes of saturated lipids. As the temperature increases the likelihood of the disruption also increases. In the liquid crystalline membranes formed by unsaturated lipids or after adding Chol to saturated lipids the deformations and defects created by mechanical motion of the MNPs are more likely to heal and results in decreased release. The study provides rationale for the design of such liposomes for future drug delivery applications.

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Surface-Enhanced Raman Spectroscopy in Glycated Albumin Detection

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New silver SERS-substrate was applied for glycated albumin (GA) selective determination using phenylboronic acid. The surface of the substrate has flaked structure with different fractions of particles: from 50 nm to 2 μm. This heterogeneous structure leads to Raman signal increase. To produce selectivity the surface of SERS-substrate was modified with 4-mercaptophenylboronic acid (4-mPBA). Different saccharides (Fru, Glc, Suc, Dex) were taken as a model compounds for the glycated proteins selective determination. They contain cis-diol groups that form six-membered ethers with boronic acid. Main differences in the SERS-spectra of sugar/glycated albumin treated SERS-substrate and control are referred to phenylboronic acid vibrations (999, 1016, 1072 and 1590 cm-1). Principal component analysis (PCA) and Partial Least Squares Regression (PLS-R) were used to discriminate spectra and to construct calibration curve, as well as to predict GA value in real samples of human plasma. New silver SERS-substrate modified by 4-mPBA allows quantitative determination of glycated albumin in human plasma.

The development of a fluoroquinolone delivery system with improved pharmacokinetic characteristics using β-cyclodextrin derivatives

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Keywords: drug delivery, cyclodextrins, fluoroquinolones

Despite the fast development of pharmaceutical industry, infectious diseases continue to be a major public and medical concern. According to the World Health Organization, there is still a great request for novel drug formulations with increased clinical efficiency against bacterial infections. For this purpose, the growing field of nanobiotechnology provide a plenty of polymeric nanocarriers such as natural and synthetic polymers, although the use of some of them is limited to the high price or low biocompatibility.

β-cyclodextrin derivatives that are approved by FDA are known to enhance the drug's solubility, stability and bioactivity as well as decrease the adverse effects by the «guest-host» complex formation [1]. In this work we propose oligomeric and polymeric colloidal structures based on β-cyclodextrin derivatives for the creation of the novel drug delivery system. It is supposed that these novel nanomaterials might enhance the effect on drug's physical chemical properties and propose controlled release of bioactive molecule.

Earlier, we have studied the prospects of β-cyclodextrin derivatives as carriers for antibacterial drugs fluoroquinolones [2]. The understanding of complex formation mechanism and the types of additional stabilizing interactions between the ligand and the drug is essential fot the development novel nanocarriers. These oligomeric or polymeric materials based on β-cyclodextrin derivatives seem to be promising for the creation of the novel drug delivery system with controlled release [3].

In this work the complex formation of β-cyclodextrin derivatives and its synthesized oligomeric particles with moxifloxacin, levofloxacin and ciprofloxacin was analyzed by FTIR-spectroscopy and UV-spectroscopy. It is shown that depending on the β-cyclodextrin's substituent and the molar excess of crosslinking agent the physical-chemical properties of its oligomers and the inclusion complexes with fluoroquinolones vary dramatically. According to obtained data, K_{dis} values of 10^{-6} M can be reached that is essential to develop the drug delivery systems with controlled release. It is demonstrated that the complex formation slows down the release of fluoroquinolones by equilibrium dialysis with and without the protease enzymes. The susceptibility of *E.Coli* 669 strain to fluoroquinolones was observed in liquid and solid media. The minimum inhibition concentration was determined by *in vitro* studies and it was found that β-cyclodextrin derivatives and its oligomers affect greatly the growth of bacteria that make these nanocarriers promising for the development of novel drug delivery systems.

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Actin cytoskeleton functioning in neurons

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Keywords: neurons, tropomyosin isoforms, actin.

Actin cytoskeleton plays a key role in such processes as cell division; formation of lamellopodia, cell processes and protrusions; maintaining cell shape; cellular transport and mobility. All these processes are extremely important for cell functioning and viability. The key role in the discrimination of actin functions is assigned to tropomyosin. Sixteen isoforms of this protein are expressed in neural tissue. However, the details of a mechanism for different isoforms of tropomyosin to cause morphological or functional changes in neurons have not been revealed. In our work we investigate structural and functional properties of different neuronal tropomyosin isoforms. We revealed that many properties of this isoform can depend from their molecular weight. We showed that the thermal stability of the N-teminal part Tpm HMW isoforms was lower than for LMW isoforms. The HMW isoforms had comparable thermal stability with the exception of Tpm1.6. The temperature of the 1-st calorimetric domain was on 3 – 4 ºC higher in the case of Tpm1.6 compared to other HMW isoforms. The enthalpy of this domain differed at 2.5 – 4.5 times. Unlike HMW isoforms, LMW isoforms differ much more in their amino acid sequence. It should be noted, that despite these differences, the temperature of the 3-rd calorimetric domain of the studied LMW isoforms was comparable. But the enthalpy of the 3-rd domain was different between isoforms. Thus, we showed that the structural properties of Tpm molecules are highly dependent on the amino acid sequence encoded by alternatively spliced exons. We were measured parameters of Tpm affinity to F-actin and the thermal stability of tropomyosin-F-actin complexes. It was found that the affinity of HMW tropomyosin isoforms to F-actin is much higher than the affinity of LMW isoforms. Interesting data were obtained by registration of the temperature dependence of the light scattering. Tpm1.6 have the least stability of the Tpm-F-actin complexes, vice versa the highest stability of the complexes was observed in the case of Tpm1.5 and Tpm4.2 neuronal isoforms. The denaturation of Tpm-actin complexes occurs primarily due to the melting of the C-terminal part of the Tpm molecule. Also, were obtained data on the bending stiffness of the reconstructed thin filaments decorated with the different Tpm neuronal isoforms. As in the case of the Tpm-actin complexes thermal stability, no unambiguous correlations between the bending stiffness of the microfilament and the size of the Tpm isoforms (HMW or LMW) could be detected. However, we can conclude that the thermal stability of the Tpm-actin complexes has a good correspondence with the bending stiffness of the microfilament. Another important part of the work was experiments in cell cultures. On MIN-6 and 1.1B4 cell cultures, expressive data were obtained. The expression of various tropomyosin isoforms led to changes in the shape of cells and the formation of lamellopodia and structures similar to neurites. This result indicates that Tpm molecules can determine the structure of the actin cytoskeleton and can induce the formation of various structures using microfilaments. We were also carried out experiments on the colocalization of various TPM isoforms with myosin I. It's turned out that some tropomyosin isoforms can be located near to this motor, and some are not. For example, Tpm 3.1 is absent in structures near myosin I, however, for Tpm1.5 there are structures in which it is located near to myosin I. Our data confirm the theory that tropomyosin isoforms can discriminate the functions of different myosin motors.

Summarizing the results of all our experiments, we can conclude that Tpm able to determine the architecture of cells by regulating the properties of actin filaments.

Co-Symposium “Extremophiles"

Microbiom International Space Station

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Keywords: International Space Station, Microbiome, Built environment

An important part of planning future long-term space flight missions (e.g. to Mars) is the development of strategies to safely manage the indoor microbiome of spacecraft, which represent completely isolated habitats. Since microorganisms pose a potential hazard for crew and spacecraft material, it is critical to assess microbial population dynamics and also to detect eventually developing resistances within the microbiota of such special closed systems. The best model system to date to investigate the microbiome of a confined habitat with constant human occupation in space is the International Space Station (ISS).

To contribute to the big picture of the ISS indoor microbiome in addition to already existing data, we have been analyzing samples from the Russian ISS module for 20 years
with a focus on extremotolerent bacteria and various fungi.

We assessed the cultivable microbiota of these samples via a broad range of cultivation assays and analysed their extremotolerant potential. Additionally, we assessed the microbial community of these samples via Next Generation Sequencing (NGS).

Besides confirming the presence of a broad variety of microorganisms on board the ISS, we were able to prove the presence of archaeal signatures on board the ISS. Elucidating the microbial population dynamics of the ISS can not only be used for spaceflight purposes but also help understanding possible risks in confined habitats on Earth, as e.g. industrial clean-rooms.

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Extremophiles in the subglacial Antarctic Lake Vostok

Bulat Sergey

Novel determinants of extracellular electron transfer underlying exo-electrogenesis and biogenic crystallization of magnetite in a thermophilic bacterium *Carboxydothermus ferrireducens*

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Keywords: extremophiles, multiheme cytochromes, Fe(III) reduction, electrogenesis, extracellular electron transfer

Extracellular electron transfer (EET) is a unique physiological feature of prokaryotes supposed to evolve among first respiratory processes on hot anoxic early Earth. In modern thermal sedimentary environments, such manifestations of this feature as biogenic transformation of Fe minerals and direct interspecies electron transfer put into use high-potential but hardly accessible insoluble electron acceptors, drive interspecies electron transport and thus energize complex microbial communities. Information on thermophiles capable of EET remains scarce, especially concerning their most abundant Gram-positive morphotype. We report formation of big magnetite crystals with unusual ultrastructure from amorphous mineral ferrihydrite, induced by Fe(III) reducing cultures of a Gram-positive bacterium *Carboxydothermus ferrireducens*, as well as exoelectrogenic activity of this organism. Confocal and scanning electron microscopy studies revealed that these processes could be driven by EET from cells adhered on insoluble electron acceptors. Genome analysis retrieved determinants of two EET strategies – 17 genes of *c*-type multiheme cytochromes with predicted outer cell surface localization and a cluster containing complete gene set for pili formation. Expression profiling of these cytochromes with shotgun proteomic technique across different cultivation conditions revealed three multihemes, which are strongly upregulated in ferrihydrite-reducing or electrogenic cells. These putative secreted cytochromes are not homologous to previously reported components of porin-cytochrome complexes, determining EET to Fe(III) oxides. Two cytochromes were upregulated in response to both ferrihydrite and electrode supplied as the electron acceptors, and one additional was only upregulated in ferrihydrite-growing cells, indicating peculiarities of EET pathway to this mineral. The most abundant and constitutive outer surface multiheme OmhA was purified to homogeneity and biochemically characterized. Pure reduced OmhA cytochrome could be reoxidized by soluble Fe(III) forms, as well as by insoluble Fe(III) form ferrihydrite (midpoint potential varying between +200 and -100 mV). Redox titration of purified OmhA revealed that the cytochrome is redox-active in the potential interval of +150 ÷ -200 mV. The potentiometric titration curve is described by Nernst equation for two one-electron centers with E_m values of +27 and -59 mV. These data correlate with the determined interval of OmhA redox-activity and support the suggestion about physiological role of OmhA as the terminal oxidoreductase, interacting directly with extracellular insoluble Fe(III) forms. Altogether, our results broaden the diversity of known extracellular electron transferring proteins with multihemes, not related to porin-cytochrome complexes and even encoded separately from another possible determinants of EET.

The work was supported by the RSF project 17-74-30025.

Insight into Extremophilic Hydrocarbonoclastic Fungi for Sustainable Development of Oil Industry

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Keywords bioconversion; mycoremediation; fungal biodiversity; oil industry; sustainability

The increasing global demand of fuels in addition to the reduction of conventional crude oil reserves has generated a greater interest on the exploitation of unconventional crude reserves. Few publications are focused on petroleum biotechnology and the applications of microorganisms in the oil industry, such as extremophilic hydrocarbonoclastic fungi, "a broad group of cultivable microorganisms which live optimally under extreme conditions, characterized by having a high ability to grow using hydrocarbons as a sole carbon source and energy" (Naranjo et al., 2019). This unexplored area of applied mycology is addressed to the degradation or bioconversion of heavy crude oil (HCO) and extra-heavy crude oil (EHCO), a type of crude that contains elevated amounts of asphaltenes, high-molecular-weight compounds with low bioavailability and limited susceptibility to be biotransformed. The potential applications of these promissory extremophilic hydrocarbonoclastic fungal strains in mycoremediation and EHCO bioupgrading processes to promote the sustainable development of the petroleum industry will be discussed.

Lipolytic enzymes from permafrost microorganisms

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Keywords: lipolytic enzymes, permafrost, metagenome, autotransporter

The unique microbial community of Siberian permafrost is adapted to low temperature, limited content of organic matter, small amount of liquid water and other extreme conditions. Structural and functional studies of the proteins from permafrost microorganisms are conducted in order to reveal molecular mechanisms of their adaptation and to assess ability to cope with various environmental challenges. We have expressed and characterized several lipolytic enzymes from *Psychrobacter cryohalolentis* K5T with the unique properties including activity in a broad temperature range and, in some cases, enhanced stability. Particularly, Lip1Pc is a typical cold-active protein that is rapidly inactivated at increased temperature, while EstPc and Lip2Pc possess relatively high thermal stability. A new autotransporter AT877 from *P. cryohalolentis* with an esterase passenger domain was utilized for the construction of an autodisplay system. We have fused the coding sequences of its α-helical linker and translocator domain with those of esterase EstPc and other heterologous passengers and demonstrated that these proteins were successfully displayed at the surface of the recombinant *E. coli* strains.

Furthermore, as a result of the construction and screening of metagenomic DNA libraries from the permafrost soil sample we have obtained and characterized new esterases belonging to the HSL family of lipolytic enzymes. We have demonstrated that PMGL2 is an esterase which is active in a broad temperature range, with an optimum at 45°C. Its amino acid sequence includes a new GCSAG motif with a putative catalytic serine residue. PMGL3 displays temperature optimum at 30°C. Incubation at 50°C led to almost complete inactivation of PMGL3, while PMGL2 preserved 65% of activity. The results of the study demonstrate that permafrost microorganisms contain lipolytic enzymes with various activity profiles and possess considerable biotechnological potential.

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Cloning, expression, and characterization of novel chitinases from extremophilic bacteria

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Keywords: extremophilic bacteria, chitinase, Chitinispirillum alkaliphilum, candidate phylum BRC1, metagenome

We expressed putative endochitinases from two extremophilic bacteria in *Escherichia coli*, purified and functionally characterized recombinant enzymes. *Chitinispirillum alkaliphilum* ACht6-1 is a fermentative haloalkaliphilic bacterium from a hypersaline alkaline lake, growing exclusively with insoluble chitin as the substrate. Genome analysis revealed two putative secreted endochitinases, Chit796 and Chit1882, containing chitin-binding domains and, respectively GH18 and GH19 catalytic domains. Functional characterization of recombinant enzymes confirmed their endochitinase activities towards amorphous chitin and synthetic soluble substrate, 4-MU-β-D-N, N′, N″-triacetylchitotriose (381 U/mg). For Chit796 enzyme the maximum activity was observed at 60°C and pH 7-8, the activity increased with increasing NaCl concentration up to 3.5M. The second enzyme was most active at 60-70°C and pH 7-8. Both enzymes were able to bind to insoluble chitin.

Another chitinase was identified in the complete genome of uncultured bacterium of the candidate phylum BRC1, assembled from metagenome of the microbial community of a 2-km-deep subsurface thermal aquifer in Western Siberia, Russia. Genome analysis of this bacterium revealed a complete set of enzymes that could enable utilization of chitin, including a GH18 family endochitinase BY40_1901, predicted to carry N-terminal secretion signal. The corresponding gene was cloned directly from environmental DNA sample and expressed in *Escherichia coli.* Chitinolytic activities of recombinant enzyme were evaluated with synthetic soluble substrates. High hydrolytic activities were observed with the endochitinase substrate 4-MU-β-D-N,N′,N″-triacetylchitotriose (287 U/mg) and with 4-MU-diacetyl-β-D-chitobioside (342 U/mg), while much lower value was detected with 4-MU-N-acetyl-β-D-glucosaminide (0.7 U/mg). This substrate specificity pattern is consistent with the predicted endochitinase activity of BY40_1901. Considering the temperature dependence, the maximum activity was measured at 50°C, that is close to the temperature of the aquifer.

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POSTERS ABSTRACTS

Minichaperone-based fusion system for stabilization of hydrophobic proteins in stably soluble forms.

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Keywords: minichaperone; fusions; stabilization of insoluble hydrophobic proteins.

In this work, the new fusion system is described, able to maintain in soluble stable form initially insoluble proteins. For the proof of principle, two initially insoluble proteins were chosen, E6 from human papilloma virus (HPV) type 16, and N-terminal fragment of E2 from hepatitis C virus (HCV). Both these proteins are considered as candidates for corresponding therapeutic vaccines owing to their immunogenicity, and both cannot be used in this capacity because they are hydrophobic and practically insoluble in native buffers. In the new fusion system, both HPV E6 and HCV E2 are expressed as inclusion bodies, but are easily renatured from urea solution and retain their solubility in native conditions through standard biochemical procedures, such as concentration, storage in solution, freezing – thawing, and lyophilization – re-dissolving. As a leader for this fusion system, a new form of minichaperone – GroEL apical domain (**GrAD**, **Gr**oEL **A**pical **D**omain) – was designed. The sequence of GroEL from thermostable organism *Thermus thermophlus* was used as a template for GrAD, which included GroEL amino acid residues 190 – 333. The final form retained the thermostability of original GroEL, and this feature can be used as a first step of purification. In GrAD sequence, the codons encoding methionine residues were substituted for those encoding leucine residues. That permits easier purification of the target polypeptide after its chemical cleavage off the fusion by cyanogen bromide. Alternatively, in the linker between GrAD and the target polypeptide the recognition site of enterokinase for enzymatic cleavage is provided. Also, two more forms of GrAD were made, and their physico-chemical properties studied. These forms allow the variability of steric interactions between GrAD and the target polypeptide, which considerably widens the possibilities of using GrAD as a leader in fusion systems

Studying the interaction of nicking endonuclease BspD6I and its variants with DNA

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Keywords: nicking endonucleases, restriction endonucleases, cysteine residue

Nicking endonucleases are enzymes that recognize specific 4-8-bp DNA sequences in length and hydrolyze only one strand in the predetermined position. Nowadays nicking endonucleases are widely used in different biotechnological methods but they remain biochemically poorly studied enzymes. The object of the present study is nicking endonuclease BspD6I (Nt.BspD6I) that is a large subunit of heterodimeric restriction endonuclease BspD6I from *Bacillus species* D6 strain [1]. Nt.BspD6I recognizes 5'-GAGTC-3'/5'-GACTC-3' sequence and cleaves the top strand 4 nucleotides downstream of the recognition site. Nt.BspD6I contains four Cys residues that can interact with DNA during the enzyme's functioning. According to the model of Nt.BspD6I-DNA complex Cys11 and Cys578 are located in close proximity to DNA.

We used method of selective crosslinking to study the possible interaction of Cys residues with DNA. In the frame of this approach, DNA duplex contained a disulfide group in 2'-position of nucleoside that could interact only with Cys residue. The DNA duplex contained one or two modified nucleosides in different positions: in the top strand in the recognition site; 4 or 6 nucleotides downstream of the recognition site in the top or bottom strand, respectively; or both in the top and bottom strands. In the case of wt Nt.BspD6I, the yield of the crosslinking reaction was 20%. The mutant form Nt.BspD6I(C11S/C160S) also interacted with the modified DNA but the yield of the reaction was lower. Thus, we suppose that Cys residues of the C-terminal domain (in the positions 508 and/or 578) can interact with DNA at the stage of complex formation. We performed comparative analysis of the ability to bind and hydrolyze DNA for wt Nt.BspD6I and Nt.BspD6I(C11S/C160S). The obtained results confirmed that Cys residues of the C-terminal domain contribute to the proper functioning of the enzyme.

To clarify the mechanism of Nt.BspD6I functioning we studied the interaction of the enzyme with fluorescently labeled 19 b.p. DNA duplexes using stopped-flow technique. The analysis of the obtained curves revealed that Nt.BspD6I binding with DNA includes at least two different stages that can relate to non-specific binding and DNA bending. The process of DNA hydrolysis also consisted of several stages that can reflect the introducing of the nick into DNA and further dissociation of the DNA-protein complex.

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Analysis of an ellagitannin-degrading enzyme produced by Aspergillus niger GH1

Aguilar Cristoba

Modeling of substrate binding with L,D-transpeptidase 2 from *Mycobacterium tuberculosis* by advanced metadynamics

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Keywords: *Mycobacterium tuberculosis*, L,D-transpeptidase, peptidoglycan, meso-diaminopimelic acid, metadynamics

While in most bacteria penicillin-binding enzymes D,D-transpeptidases play the major role in the cell wall peptidoglycan cross-linking catalyzing formation of the so-called 4-3 bonds between 4th D-Ala and 3rd meso-diaminopimelic acid (m-Dap) residues of different chains, in *Mycobacterium tuberculosis* the most of cross-links are formed by L,D-transpeptidases (LdtMt), that catalyze formation of 3-3 cross-links between two 3rd m-Dap residues. L,D-transpeptidases, in contrast to D,D-transpeptidases, are not sensitive to widely used penicillins and cephalosporins [1,2], and are considered as attractive molecular targets searching for novel antitubercular agents.

In this work interaction of L,D-transpeptidase 2 from *M. tuberculosis* with natural peptidoglycan fragment that consists of both glycan (N-acetyl-D-glucosamine [NAG] and N-acetyl muramic acid [NAM]) and tetrapeptide (L-Ala-γ-D-Glu-m-Dap-D-Ala) parts and contain either C-terminal carboxyl groups of γ-D-Glu(COO^-) and m-Dap(COO^-) residues or their amides, was studied using advanced molecular modeling. The force field parameters were obtained for natural peptidoglycan fragment NAG-NAM-[L-Ala-γ-D-Glu-m-Dap-D-Ala]-NAG that include γ-D-Glu and m-Dap residues in both carboxyl and amide forms: γ-D-Glu(COO^-/$CONH_2$) and m-Dap(COO^-/$CONH_2$). Energetics of the active site lid opening process that control substrate delivery to the active site was evaluated using the well-tempered metadynamics. The system's collective variables which describe formation of the enzyme-substrate complex were selected and free energy surface (FES) for the substrate binding in the active site was built. Energetically more favorable formation of enzyme-substrate complex in case of amide substrate was observed compared to the corresponding substrate bearing free carboxyl groups. The results of molecular modeling are supported by the experimental data.

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Peculiarities of HIV-1 associated MHC-I-peptide-TCR complexes formation

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Keywords: TCR-pMHC interactions, T-cell activation, immunology, TCR-mediated signaling, HIV-1.

Search for molecular bases of T cell activation triggered by TCR-pMHC interaction is the keystone of the adaptive immune response against HIV-1 infection. We suppose that structural changes at the TCR-pMHC-I interface can affect the parameters of the pMHC-I recognition by T cells and determine the TCR-mediated signaling kinetics and the efficiency of the cytotoxic response. Based on these considerations, we hypothesized that only a certain combination of TCR, peptide and MHC-I variants and a certain mode of their interaction determines the efficacy of the interaction of TCR with pMHC-I and provides rapid kinetics and selectivity of T cell-mediated cytotoxic response against virus-infected target cells in the result. Part of our work was dedicated to pinpointing on some dynamical features of pMHC-TCR binding/unbinding process that may correlate with known signaling propensity of systems under consideration. We utilized steered molecular dynamics simulations on 12 test systems to show that one such feature may be manifested as the disproportion of TCR interactions with a1 and a2 alpha-helices of MHC directly influenced by harbored peptide. This observation can serve as not only a heuristic model for sorting out signaling complexes from silent ones but also lead to clarifying the basic mechanisms of T-cell activation. To experimental confirmation of computational algorithm described above, we design the genetic construction for production of TCR and MHC-I in *E. coli* and *D. melanogaster* S2 cells respectively. It was shown that TCR D3 produced in the bacterial system of *E. coli* has a tertiary structure and high thermal stability (melting point - Tm = 62.5 ° C). Thereby, TCR D3 is suitable for crystallization trials as an independent object and in combination with the corresponding pMHC-I-peptide pair. We determine the kinetic and thermodynamic properties of TCR-pMHC-I interaction. This work was performed in the frame of RFBR Grant # 17-54-30025. Calculations were carried out using the equipment of the shared research facilities of HPC computing resources at Lomonosov Moscow State University supported by the project RFMEFI62117X0011.

The basic kinetic model of the oscillattions in biocatalytic processes

Bykov Valery

Molecular modeling of penicillin acylase mutant with increased specificity to bromoacetamide as acyl donor

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Keywords: rational design, penicillin acylase, halogen-substituted acyl donors, molecular dynamics

As was shown recently, the halogen-substituted acetamides can be used as acyl donors in penicillin acylase-catalyzed reactions [1]. It opens a way for synthesis of new derivatives of beta-lactam antibiotics using approaches of "click-chemistry" [2,3]. However affinity and catalytic activity of the wild type penicillin acylase from *E. coli* toward haloacetamides as acyl donors in aqueous medium is quite limited what constricts the effectivity of acylation of beta-lactam antibiotics nuclei.
In this work acyl transfer in aqueous medium catalyzed by the wild type penicillin acylase from *E. coli* and its bV56W+bF256R mutant using bromoacetamide as acyl donor was studied using advanced molecular modeling. Mutant structure was prepared *in silico* using classic molecular dynamics. The force field for bromoacylserine residue was parameterized by QM calculations. Full-atomic structures of both bromoacylenzymes were obtained using hybrid QM/MM methods and well-tempered metadynamics, complex collective variables (CVs) were chosen to describe main catalytic stages at the formation of acylenzymes. Every stage was investigated in phase space of two CVs which were the distance between attacking (or leaving) nucleophile and the number of protons bonded to catalytic N-terminal serine amino group (or leaving nucleophile).
An approach of automatic searching of stationary points in selected phase space was developed and implemented in R language. The method is based on the properties of multivariate function: numerical differentiation of free energy surface for each collective variable and testing first- and second-order derivatives against special criteria with desirable tolerance to discover any stationary points; this is especially useful in case of high-dimension phase spaces. Implemented approach was used to determine energy profiles of elementary reaction stages and compare energetics of acyl transfer catalyzed by two forms of penicillin acylase from *E. coli* (wild type and bV56W+bF256R mutant).
This work was supported by RFBR (grant 18-29-13060)

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In search of transport network to trinuclear copper centre of two-domain laccases

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Keywords: two-domain laccases, crystal structures, T2/T3 copper site, channels

Laccases are blue multicopper oxidoreductases highly important for biotechnology and environmental remediation. These enzymes acting on various phenol compounds, aromatic amines, certain dyes, and metal cyanide complexes, so far found in higher plants, some insects, fungi and bacteria. Along with the typical three domain laccases (3DLac) bacteria produce two-domain laccases (2DLac) which are functionally active in the neutral and alkaline pH area, thermostable and stable against inhibitors. Laccases contain four Cu atoms per molecule organized into three sites: T1, T2 and T3. Mononuclear T2 copper ion and two T3 copper ions ($Cu3_{\alpha}$ and $Cu3_{\beta}$) form a trinuclear center (TNC). The catalytic mechanism in T1 site of two-domain laccases has been intensively studied, but dioxygen/water transport to/from TNC is not yet fully understood.

Here we present the comparative analysis of crystal structures and catalytic properties of recombinant 2DLac from *Streptomyces griseoflavus* Ac-993 (SgfSL) and four mutant forms with replacements of the two highly conserved for 2DLac amino acids, located in the presumable T3 "oxygen" channels. The side chain of Ile170 significantly narrows one of the calculated channels and His 165 closes the shortest route from the protein surface to the cavity between $Cu3_{\alpha}$ and $Cu3_{\beta}$.

As a result we obtained catalytically "dead" ferments at substitutions of His165 to Phe and Ile170 with both Ala and Phe. The activity of His165Ala SgSL is slightly higher than wild type.

We suggest that side chain of His165 can work like a "gateway" of T3 "O_2-channel" and selectively pass to the TNC of 2DLac dioxygen and arrest other molecules (for example, inhibitors). In addition "close" position of imidazole ring of His165 may be essential for correct dioxygen position in TNC during reduction process in 2DLac. The results of replacing Ile170 with Ala and Phe indicate the absence of the second supposed channel leading to TNC. We assume that side chain of Ile170 maintaining the position of the His157 imidazole ring which coordinates $Cu3_{\alpha}$ and what influences to molecular oxygen reduction reaction.

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Catalytic processing of human type receptors by H5N1 Influenza neuraminidase using molecular modeling

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Keywords: Influenza, neuraminidase, oligosaccharides, metadynamics

Detailed understanding of neuraminidase catalysis could allow to model and predict the effect of various mutations on enzyme's substrate specificity and design its effective inhibitors. This issue is especially critical regarding the highly pathogenic avian influenza viruses (HPAIV), including H5N1 strain, causing disease outbreaks not only in the bird population, but also in humans (WHO data [1]). Viral neuraminidase selectively hydrolyzes sialylated glycans, being one of the key HPAIV elements ensuring receptor specificity for a particular strain which varies significantly between avian and human populations thus providing a natural barrier for direct transmission. The study of H5N1 neuraminidase reactivity towards human receptors is paramount since the exact scheme of the mechanism and the evaluation of the catalytic stages are still lacking.

The interaction of influenza neuraminidase with oligosaccharide fragments of sialylated glycans is a complex process determined by the multidimensional phase space of various conformations [2]. Thus, we have used comprehensive analysis of glycan's conformational phase space to avoid unsystematic interpretation of the particular conditions of modeling as in one of the studies [3] authors failed to obtain an adequate model of the substrate specificity of H5N1 neuraminidase known to possess higher activity toward avian receptors than human ones comparing to seasonal strains [4]. The study has been performed using 6'sialolactose (α-Neu5Ac-2,6-β-Gal-1,4-β-Glc-O-Me) as a carbohydrate fragment of the receptor. Structure of a Michaelis complex has been revealed by using nonparametric Bayesian clustering of glycan structures obtained from 500 ns molecular dynamics simulation on the MSU Lomonosov 2 supercomputer [5]: most populated cluster permitting the distortion of sialic acid ring structure has been taken into account. Formation of a covalent glycosyl-enzyme complex has been evaluated applying multidimensional free energy calculation by combined quantum-mechanical/molecular-mechanical metadynamics. The results, together with our previously determined free energy profile for 3'sialolactose hydrolysis demonstrate how differences in the chemical structure of oligosacharides affect catalytic efficiency and convenient for evaluating adaptive mutations that evolve human tropism.

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Nuclear Spin Catalysis in Biomolecutar Motors: Premises and Promises

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Keywords: biocatalysts, magnetic-isotope effect, nuclear spin catalysis, molecular motors

Cells are composed from atoms of chemical elements, many of which have magnetic and nonmagnetic stable isotopes. In physics and chemistry, magnetic isotope effects (MIEs) have long been known for a number of magnetic isotopes, among them ^{13}C, ^{17}O, ^{29}Si, ^{33}S, ^{73}Ge, and ^{235}U (Zeldovich, Buchachenko, Frankevich, 1988). Not long ago, MIEs have been discovered in experiments with living cells. In studies of effects of different isotopes of magnesium, magnetic ^{25}Mg and nonmagnetic ^{24}Mg, on the post-radiation recovery of the yeast *Saccharomyces*, irradiated by short-wave UV light or X-rays, it has been revealed that the recovery process of the cells, enriched with the magnetic ^{25}Mg, proceeds two times faster than the post-radiation recovery of the cells, enriched with the nonmagnetic ^{24}Mg. In the experiments with another cell model, bacteria *E. coli*, it has been found that bacterial cells adapt essentially faster to the growth media enriched with magnetic ^{25}Mg compared to the media enriched with the nonmagnetic isotopes of magnesium. Besides, the cells enriched with ^{25}Mg demonstrate the reduced activity of the important antioxidant enzyme, superoxide dismutase, by comparison to the cells enriched with the nonmagnetic ^{24}Mg. Thus, it has been discovered that living cell perceive the nuclear magnetism (Avdeeva, Koltover, 2016; Avdeeva et al., 2019). Furthermore, MIEs have been revealed in studies of the most important molecular motor of cell bioenergetics, myosin isolated from smooth muscle. The rate of the ATP hydrolysis, driven by myosin, is 2.0-2.5 times higher with ^{25}Mg than that with the nonmagnetic ^{24}Mg or ^{26}Mg (Koltover et al., 2016). The similar MIE has been revealed with zinc. While Zn^{2+} performs the cofactor function less efficiently than Mg^{2+}, the rate of the ATP hydrolysis driven by myosin is 40-50 percent higher with the magnetic ^{67}Zn as compared to the nonmagnetic ^{64}Zn or ^{68}Zn (Koltover, Labyntseva, Kosterin, 2018). Moreover, the beneficial MIE of ^{25}Mg has been discovered in the reaction of ATP hydrolysis catalyzed by mitochondrial H^+-ATPase, isolated from yeast cells and reconstituted into the proteoliposome membrane. On its own, factual evidence of MIE unambiguously indicates that there is a spin-selective rate-limiting step, the “bottle-neck” in the chemo-mechanical cycle of the enzyme, that is accelerated by the nuclear spins of ^{25}Mg or ^{67}Zn. The nuclear spin catalysis in the molecular motors may be explained as follows. The energy released during ATP hydrolysis is not large enough to trigger the electron-conformational excitation of the macromolecule into the singlet exited state. It is sufficient to trigger a low-level triplet state. However, the transition from the ground state ($S = 0$) into the triplet state ($S = 1$) is forbidden by the spin conservation law. The magnetic isotope’s nuclear spin eliminates the spin ban providing the required spin conversion into the triplet state, thereby providing the acceleration of the chemo-mechanical cycle of the enzyme. The alternative explanation of the nuclear spin catalytic effects suggests a virtual radical-ion pair in the enzyme’s active center, which interferes with the ATP hydrolysis. Creating the spin ban, the magnetic isotope prevents the undesired reverse reaction of ATP synthesis thereby promoting the direct ATP hydrolysis reaction. The hypothesis about the key-role of such a virtual radical-ion pair in ATP synthesis at oxidative phosphorylation was stated about 50 years ago (Blumenfeld, Koltover, 1972). Although detailed mechanisms of ability of the biocatalysts to perceive the nuclear magnetism require further investigations, there are the grounds to believe that this new field, nuclear spin catalysis, highlights promising venues for future research with possible applications of the stable magnetic isotopes in medicine for creating novel anti-stress drugs including the low-toxic anti-radiation protectors.

Effects on N-terminus structure on stability and catalytic properties of formate dehydrogenase from yeast *Ogataea parapolymorpha*

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Keywords: formate dehydrogenase, N-terminus, protein stability

Formate dehydrogenase (FDH, EC 1.2.1.2) catalyzes the oxidation of formate ion to carbon dioxide coupled with reduction of $NAD(P)^+$ to NAD(P)H, which allows its use to regenerate the cofactor in the processes in which NAD(P)H is consumed. NAD^+-dependent FDHs from various sources are actively studied in our laboratory.

Previously in our laboratory, the FDH gene from the yeast *Ogataea parapolymorpha* DL-1 (OpaFDH) was cloned and expressed in *E.coli* cells. However, since at the N-terminus of the native form of the enzyme is a lysine residue, which, according to the N-end rule, should reduce the stability of the protein; when cloned, an additional triplet encoding glycine residue was added to the gene sequence at the N-terminus. In addition, by analogy with bacterial FDHs, a mutant with an alanine residue at the N-terminus was obtained. Thus, two mutant forms of FDH were obtained from the yeast *Ogataea parapolymorpha* DL-1 with additional glycine and alanine residues at the N-terminus (OpaFDH1 and OpaFDH2 respectively). From the data obtained during cultivation in *E.coli* cells, it can be concluded that OpaFDH2 has a higher activity yield (1,5 times higher than in the case of OpaFDH1). Further, according to the method developed earlier in the laboratory, the stages of isolation and purification of OpaFDH1 and OpaFDH2 were carried out. Enzyme samples in a high purified state were used to study temperature stability and catalytic properties. It was found that the form of OpaFDH2 is 2-6 times more stable at high temperatures than OpaFDH1, while both forms are concede in this parameter only to FDHs from bacteria *Pseudomonas* sp.101 and *Staphylococcus aureus*. Studying of kinetic parameters showed that Michaelis constants for both forms of OpaFDH do not differ noticeably.

The work was supported of the Russian Foundation for Basic Research (grant 18-34-20098).

Structural and functional characterization of a new member of the HSL family from Siberian permafrost microbial community

Krukova Maria

Role of CopC from Thioalkalivibrio paradoxus as a copper chaperone

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Keywords: Thioalkalivibrio, CopC, copper, chaperone, TcDH.

CopC is a small periplasmic protein from haloalkaliphilic bacteria of the genus Thioalkalivibrio. This CopC differs from other well-known CopC proteins by the presence only Cu(II) binding site. By tryptophan fluorescence quenching method we showed that CopC can bind only one Cu(II) ion.

CopC belongs to the high-affinity CopC subfamily (C0-2) with conserved N-terminal His1-Xxx-His3 motif [Lawton et al., 2016]. It is known that CopC proteins can play the role of copper chaperon [Hussain et al., 2007]. In this regard Thiocyanate dehydrogenase (TcDH) as a copper containing protein from the same bacteria was chosen. When the bacterium Tv. paradoxus ARh1 grows with thiocyanate, the expression level of CopCD genes increases in parallel with that of TcDH genes. Genes of copper-binding proteins CopCD are located before the TcDH gene in Thioalkalivibrio genomes.

TcDH apo form (containing no copper ions) can be activated by the Cu(II) sarurated CopC more effective than that of Cu(II) ions at the same concentration.

By tryptophan fluorescence quenching assay we revealed the complex formation (TcDH and CopC) with stoichiometric ratio 1:2. After complex separating using gel-filtration the increasing of copper content in the molecule of TcDH with decreasing of that in the molecule of CopC was observed.

The activity of copper-rich TcDH is also increased in comparison with TcDH apo form. The data obtained indicate the possibility of TcDH to form a complex with CopC with the subsequent transfer of copper ions to the TcDH molecule.

These data confirm that CopC can play the role of chaperone responsible for incorporation of copper into the TcDH active site.

Antibody-based biocatalysts: how to predict selectivity and improve reactivity toward organophosphorus compounds.

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Keywords: catalytic antibodies, organophosphorus compounds, covalent catalysis, stereoselectivity

Today one of the most challenging directions in the generation of novel biocatalysts is a simplification of the screening approaches for the well-defined substrate stereoselectivity or changing/improving the catalytic activity. Traditionally, the basic strategy for biocatalysts engineering with desired properties are rational design based on the crystal structures analysis or combinatorial biology approaches. X-ray structural models are widely used for the direct evolution of molecules, but these structures represent only one variant of the protein state without taking into account conformational changes upon interaction with the substrate. Otherwise, combinatorial methods are limited by selection options for the catalytic activity and take long-term efforts. Modern computational tools allow simulating of the chemical reactions in multicomponent biological systems and could be useful as an alternative to these approaches.

In present work, we confirm that QM/MM calculation approach is a viable tool for the prediction of the aryl-phosphonate specific abzyme stereoselective properties. Additionally, for the most reactive catalytic antibody A17, QM/MM calculations allow us not only increase the efficacy of interaction with the selected substrate but also change the basic mechanism of covalent catalysis. New *in silico* algorithms for virtual screening of biocatalysts opens broad prospects for the redesigning activity and specificity of immunoglobulin-catalyzed reactions.

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The ectoine degradation pathway in halotolerant methanotrophs

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Keywords: methylotrophs, *Methylomicrobium alcaliphilum* 20Z, ectoine degradation

The ability to synthesize ectoine (1,4,5,6,tetra-2-methyl-4-pyrimidonecarboxylic acid) is an important trait for methylotrophic bacteria that populate marine and terrestrial habitats (Reshetnikov et al., 2011). To maintain an osmotic equilibrium with the surrounding medium, the halophilic and halotolerant methylotrophs synthesize ectoine accumulating it at very high concentration thus providing them ability to grow at salinities up to 2 M NaCl. Besides osmotic balancing, ectoine exerts the beneficial effect on microbial interior stabilizing protein folding and protects whole cells against various stresses such as UV radiation or cytotoxins. Methylotrophs employ a pathway for ectoine biosynthesis similar to that described in halophilic heterotrophs involving the EctABC enzymes which are highly conserved among ectoine-producing bacteria.

Bacteria undergoing the variation of medium salinity can change intracellular ectoine level via different mechanisms. Besides regulation at the levels of the *ectABC* transcription and activities of enzymes, halophilic bacteria can excrete ectoine in surrounding medium or/and decompose up to the central metabolites upon decrease of external salinity. We examined the pathway of ectoine degradation in the halotolerant methanotroph *Methylomicrobium alcaliphilum* 20Z. The *Mm. alcaliphilum* genome codes putative enzymes necessary for ectoine breakdown: ectoine hydrolase (DoeA), Nα-acetyl-L-2,4-diaminobutyrate deacetylase (DoeB), diaminobutyrate transaminase (DoeD) and aspartate-semialdehyde dehydrogenase (DoeC). The genes for these proteins are located in operon *doeBDAC* and display some homology to the ectoine metabolizing enzymes of *Halomonas elongata*. The function of the ectoine degrading pathway was proven by mutational analysis. The mutant strains of *Mm. alcaliphilum* with the disrupted *doeA* or *doeB* genes were obtained. Δ*doeA*-mutant accumulated more ectoine as compared to the wild type strain. The cells of the Δ*doeB* mutant grown at 6% NaCl contained Nγ-acetyl-L-2,4-diaminobutyric acid, which is the precursor of ectoine biosynthesis by ectoine synthase. The cells of Δ*doeB* exposed to the hypoosmotic shock accumulated Nα-acetyl-L-2,4-diaminobutyric acid, which is the first product of ectoine hydrolysis by DoeA and the substrate for DoeB enzyme. Thus, we proved that degradation of ectoine in methylotrophs proceeds via hydrolysis of ectoine to Nα-acetyl-L-2,4-diaminobutyric acid, followed by deacetylation to diaminobutyric acid (DoeB). Diaminobutyric acid can flow off to aspartate. The genomic analysis showed that genes for ectoine degradation pathway are present in all methylotrophs able to ectoine biosynthesis.

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The amino acid substitutions in NADPH preferring acrylyl-CoA reductase AcuI shift the enzyme specificity toward NADH

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Keywords: Acrylate metabolism, acrylyl-CoA reductase, NADH/NADPH specificity

Acrylate is a well-known compound since it widely used to produce acrylic polymers. Acrylate is generated in significant quantities through the microbial cleavage of dimethylsulfoniopropionate (DMSP), a highly abundant osmoprotectant in marine algae and phytoplankton. Acrylate can inhibit bacterial growth, likely through its conversion to the highly toxic molecule acrylyl-CoA. A number of bacteria contain acrylyl-CoA reductase (AcuI) that catalyzes the reduction of acrylyl-CoA to propionyl-CoA. AcuI enzymes from *Ruegeria pomeroyi* DSS-3 and *Escherichia coli* MG1655 have a high affinity to acrylyl-CoA and relatively high specific activity utilizing NADPH as cofactor. In several biotechnological processes the NADH-dependent enzymes is more preferential due to generally higher NADH intracellular pool. The aim of this work is to shift the cofactor specificity of the *E. coli* AcuI toward NADH and to increase the enzyme specific activity.

Analysis of the X-ray diffraction data of the enzyme crystal in the complex with NADPH published in the database showed that the negatively charged phosphate group of NADP is involved in interaction with the positive amino group of arginine 180 and 198, and also forms hydrogen bonds with serine 178 and asparagine 313 of polypeptide chain, thus determining the specificity for the NADPH. We obtained mutant variant of the AcuI enzyme from *E. coli* MG1655 with amino acid substitutions in the positions S178A, R180M, R198M and N313L. Analysis of the mutant enzyme showed a decrease in specific activity and *K*m with NADPH (*V*max = 3 U/mg of protein, *K*m = 0.15 mM), relative to the wild type AcuI (*V*max = 13 U/mg of protein, *K*m = 0.03 mm).

An amino acid substitution at position S156D which expected to determine the formation of hydrogen bonds of the carboxylic group of aspartate and hydroxyls of the ribose ring of NADH did not increased AcuI activity and specificity for NADH. Analysis of the mutant enzyme S156D revealed a decrease in specific activity and affinity with NADPH (*V*max = 0.38 U/mg of protein, *K*m = 0.11 mM) without significant changes in those for NADH (*V*max = 2 U/mg of protein, *K*m = 0.1 mM). Thus, we have obtained a number of mutant forms of the AcuI enzyme displaying changes in cofactor specificity. The resulting AcuI enzyme with new biochemical properties will serve as a basis for further investigation to understand the structure of interaction with the cofactor NADH/NADPH, and an alternative in metabolic engineering for NADPH-dependent acrylyl-CoA reductases.

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Structure and function of two types of bacterial luciferase

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Keywords: bioluminescent reaction, bacterial luciferase, multistep reaction, structure-function paradigma

The light-emitting reaction of luminous bacteria is one of the longest studied but still not the best understood bioluminescent process. Bacterial luciferase is a flavin-dependent monooxygenase, which catalyzes the reaction with formation of electronically excited intermediate through multistep process accompanied by a number of dark pathways. Based on functional features, luciferases from different bacterial species can be divided into two groups conventionally called “fast” and “slow” ones. This study aimed at revealing of the peculiarities in “architecture” of the active sites of two types of bacterial luciferases that are responsible for the preferable sequence of substrate binding and the consequent reaction kinetics.

The non-stationary kinetics of the bacterial bioluminescent reaction catalyzed by “fast” (*P. leiognathi*) and “slow” (*V. harveyi*) luciferase was studied by stopped-flow technique using analyzer SX-20 (Applied Photophysics). Modeling of the kinetic curves in Scilab program (Scilab Enterprises) was performed to define the rate constants of particular reaction steps. The molecular docking simulation was fulfilled to elucidate the binding mode of decanal, dodecanal and tetradecanal in the active site of bacterial luciferases using Autodock Vina. Also the phylogenetic analysis was made for 21 bacterial luciferase α-subunit sequences containing the active site of the enzyme to reveal the key amino acid residuals that could be responsible for functional distinctions of “fast” and “slow” luciferases.

The modeling of bioluminescence kinetics in reaction involving aldehydes with varying chain length confirmed that the aldehyde binding is a critical step for light emission rate. The molecular docking indicated that *P. leiognathi* luciferase binds tetradecanal with higher affinity than decanal, while *V. harveyi* luciferase exhibits a reversed pattern. Additionally both luciferases were found to fix the flavin-4a-hydroperoxide in a similar way in their active sites. The phylogenetic analysis of luciferase sequences revealed their splitting into two distinct groups based on highly conserved 22 amino acid residues. Two of them are involved in phenylalanine shifting near the si-face side of the isoalloxazine moiety: αIle6 (“fast”)↔αPhe6 (“slow”) and αPhe8 (“fast”)↔αLue8 (“slow”). The other three polymorphic positions form a binding platform for the re-face of the pyrimidine end: αAla74-αAla75-αCys106 in “slow” luciferases and αMet74-αGly75-αVal106 in the “fast” ones. The existence of two different sulfur-containing amino acids probably indicates an alternative flavin intermediate stabilization mechanism. Results reveal the property of bacterial luciferases to host the same biochemical reaction with critically distinct kinetics characteristics.

Investigating dynamic characteristics of the binding pocket in the influenza hemagglutinin

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Keywords: hemagglutinin, metadynamics, influenza virus.

The dynamic characteristics of the protein binding pockets can play an important role searching for new inhibitors as promising medical drugs. Potential compounds selected according to their influence on a dynamic protein structure might have a higher activity and selectivity by overcoming disadvantageous interactions within a pocket at its structural rearrangements. In case of hemagglutinin a structural rearrangement is responsible for the fusion of virus and host cell membranes, followed by the spread of viral ribonucleoproteins into a healthy cell. It is formed by the parts of the polypeptide chain that undergo significant conformational changes at the membranes fusion. Basing on X-ray crystallography data it was suggested [1] that binding of the three umifenovir molecules in three static hemagglutinin binding sites S1 can create a network of non-covalent interactions that prevent hemagglutinin restructuring at fusion to the host cell membrane. Thus, the possible stabilization of the protein structure due to binding of compounds in S1 binding site can make it an attractive target for comprehensive search of effective drugs against Influenza virus.

In this work we have investigated a dynamic behavior of S1 hemagglutinin binding sites in H3N2 and H7N9 Influenza subtypes. We have calculated the energy profiles of these pockets depending on the changes in S1 volumes using enhanced sampling by metadynamics what allowed to accelerate accumulation of statistics of rare events while controlling various system characteristics using the so-called collective variables (CV). In the case of the H3N2, the S1 volume was used as the only CV specified by the presence of water molecules within a pocket studying its dynamics in an aqueous solution. In case of H7N9 hemagglutinin the S1 binding site is known to possess a closed (without inhibitor) and an open (inhibitor bound) conformation that is determined by the position of Arg54 in the chain B: therefore additional CV taking into account positioning of Arg54 was used to perform the metadynamics. As a result free energy maps were obtained and allowed to characterize the S1 volume with a minimum energy and identify amino acid residues foremost responsible for the dynamic volume change. The sampled S1 pocket structures might serve as a framework searching for new compounds able to bind in a S1 site effectively and impair structural rearrangements of the Influenza hemagglutinin. The research was carried out using the equipment of the shared research facilities of HPC computing resources at Lomonosov Moscow State University [2].

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Fusion enzymes based on monooxygenases with internal cofactor regeneration

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key words: biocatalysts, cytochromes P450, formate dehydrogenase, fusion enzymes

NADPH-dependent monooxygenases are widely used in biotechnology processes for synthesis of different organic compounds. Such enzymes require regeneration of the expensive coenzyme NADPH to reduce its costs. For this purpose enzymes as formate dehydrogenase (FDH), phosphate dehydrogenase (PDH), glucose dehydrogenase (GDH) and glucose-6-phosphate dehydrogenase (GPDH) are commonly used. The main advantages of FDHs are wide pH-optimum (6-9.5) of activity, high thermal stability and low price of the substrate – formate ion. Most of FDHs are NAD^+-dependent enzymes and several mutant forms with changed coenzyme specificity from NAD^+ to $NADP^+$ were obtained in our lab using rational design approach.

Several genetic constructions, encoded different variants of hybrid enzymes, consisted of the genes of cytochrome P450 BM3 from *Bacillus megaterium* or phenylacetone monooxygenase (PAMO) from *Thermobifida fusca* and $NADP^+$-dependent formate dehydrogenase were created. The full size of the polypeptide chain in case of (BM3 P450 + FDH) is more than 1200 amino acid residues (more than 800 amino acid residues of cytochrome BM3 P450 and 400 amino acid residues for FDH) and the dimer construct is more than 2400 amino acid residues. The fusion enzymes of this size were prepared for the first time. All hybrid enzymes were expressed in active and soluble forms and the purification procedures were developed. All enzymes are active as FDH part and BM3 part or FDH part and PAMO part. The comparison of properties of separate enzymes and fusions is provided.

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Structural studies of formate dehydrogenases

Savin Svyatoslav

Role of Cys residue in thermal and chemical stability of formate dehydrogenase from yeast *Ogataea parapolymorpha*

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Key words: formate dehydrogenase, site-directed mutagenesis, operational stability

Formate dehydrogenase (FDH) belongs to the family of oxidoreductases. This enzyme catalyses the reaction of $HCOO^-$ oxidation coupled with reduction of NAD^+ to NADH. Due to this, FDH is used for cofactor regeneration in different commercial processes of chiral compounds synthesis. Earlier the gene encoded NAD^+-dependent formate dehydrogenase from methylotrophic yeast *Ogataea parapolymorpha* was cloned and expressed in *E.coli*. It was shown that this formate dehydrogenase (OpaFDH) had lower K_M values for formate and NAD^+. Also it was more thermally stable then formate dehydrogenases from other sources. However, OpaFDH has low operational stability.

There are three Cys residues in OpaFDH molecule and one of them is localized in the active site. It is known, that Cys oxidation can have a negative effect on stability of the protein. One mutant form of OpaFDH with replacement Cys/Ala was earlier obtained in our lab. It resulted in higher (by a factor of 100) operational stability. At the same time thermal stability of this mutant was lower compared to wt-OpaFDH. In this work, the experiments on mutagenesis of this residue were continued and mutant forms with substitutions Cys/Ser, Cys/Thr and Cys/Val were obtained. Changes C/S and C/T resulted in 100 times increase of operational stability (like in case of replacement C/A). At the same time the catalytic efficiency of the mutant forms was lower 17.5 and 87.5 times for C/S and C/T, respectively. Replacement C/V resulted in decrease of catalytic efficiency 2 times, but at the same time this mutant form was 100 times better in operational stability and 4 times more stable at 50ºC.

The work was carried out with the financial support of the Russian Foundation for Basic Research (grant 18-34-20098).

Preliminary characterization of two novel thiocyanate dehydrogenases from halophilic sulfur oxidizing *Gammaproteobacteria Thiohalobacter thiocyanaticus* HRh1^T and *Guyparkeria* sp. SCN-R1.

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Keywords: halophiles, sulfur-oxidizing bacteria, thiocynate oxidation, thiocyanate dehydrogenase.

The genomes of *Thiohalobacter thiocyanaticus* and *Guyparkeria* sp. SCN-R1, two halophilic sulfur-oxidizing bacteria (SOB) capable of thiocyanate oxidation via the “cyanate pathway”, have been analyzed with a particular focus on their thiocyanate-oxidizing potential and sulfur oxidation pathways. Both genomes encode homologues of the enzyme thiocyanate dehydrogenase (TcDH) that oxidizes thiocyanate via the “cyanate pathway” in members of the haloalkaliphilic SOB of the genus *Thioalkalivibrio*. However, despite the presence of conservative motives indicative of TcDH, the putative TcDH of the halophilic SOB have a low overall amino acid similarity to the *Thioalkalivibrio* enzyme, and also the surrounding genes in the locus were different. In particular, an alternative copper transport system *Cus* is present instead of *Cop* and a putative zero-valent sulfur acceptor protein gene appears just before TcDH.

TcDH from *T. thiocyanaticus* was purified from the periplasmic fraction of cells grown on thiocyanate and characterized.

To produce the recombinant TcDHs for further functional and structural studies the expression cassette consisted of the N-terminal His_6-tag sequence, the tobacco etch virus (TEV) protease digestion site, and the target genes. Protein expression in *E. coli* BL21Star (DE3) cells was induced with IPTG and analysed by SDS–PAGE. Protein purification was performed by two steps of metal affinity chromatography on Ni-agarose followed by gel filtration step. Both isolated enzymes exist in a solution as aggregates of different molecular weight. No characteristic activity in thiocyanate oxidation was detected after copper saturation.

TcDHs studied contains seven (*Guyparkeria*) and six (*Thiohalobacter*) Cys residues in the sequences. Therefore, potentially any helper may be necessary for the proper folding of these proteins. A gene cluster upstream of the TcDH gene in *Guyparkeria* and *Thiohalobacter* encodes protein belonging to thioredoxin superfamily. Quite probable this protein promotes correct folding TcDH. To verify this assumption corresponding genes were cloned into plasmid vector containing in the expression cassette thioredoxin as a carrier protein along with the N-terminal His_6-tag sequence and the tobacco etch virus (TEV) protease digestion site. Investigation of these recombinant proteins is in progress now.

Crystallization and structure analysis of the mutant forms of thiocyanate dehydrogenase from *Thioalkalivibrio paradoxus*

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Keywords: thiocyanate dehydrogenase, surface mutants, crystallization, X-ray analysis.

Thiocyanate dehydrogenase (TcDH) is a recently discovered enzyme which catalyzes the two-electron oxidation of thiocyanate to cyanate and elemental sulfur without utilizing oxygen as a cosubstrate. Active site of TcDH contains a unique 3Cu cluster with a novel configuration of copper ions. In solution, TcDH exists as a dimer. The 3D structure of TcDH was determined by X-Ray analysis; recombinant forms of the enzyme were obtained in *E. coli* and characterized; a hypothetical mechanism of the catalytic stage was formulated by the combined method of quantum and molecular mechanics. One of the main problems in the study of the catalytic mechanism of TcDH is the insufficiently high quality of 3D structures due to formation of twinned crystals during TcDH crystallization. Twinning prevents the determination of the exact location of copper ions in the active center of the enzyme by anomalous scattering. The aim of this work was to obtain recombinant TcDH, which does not form twins during crystallization.

TcDH forms crystals with monoclinic type of unit cell ($P2_1$) containing four molecules of the protein (tetramer) that has the tendency to form twins. It was suggested that a cleavage of the weak hydrogen and electrostatic bonds between two dimers can lead to alteration of the unit cell type and consequently to vanishing of twinning. The surface residues involved in the contacts between the TcDH subunits in a tetramer in crystals were identified in the structure and substituted. Two recombinant surface mutants of TcDH with substitutions K281A and K264, K267A were obtained in the *E. coli* cells and purified to homogeneity using two steps procedure comprising metal affinity and size exclusion chromatographies. Both mutants were dimers in a solution. After copper saturation they were active in the reaction of thiocynate oxidation. Then for each of the mutants the crystallization conditions were screened and optimized. The mutant K281A formed the crystals with unit cell belonging to $P2_1$ group of symmetry like the native enzyme and, therefore, represented the twins. The crystals of the second mutant K264A, K267A did not reveal the diffraction patterns, and the further experiments are being carried out to optimize the crystallization conditions and to gain well-ordered crystals of this mutant with high resolution.

Nucleoside phosphorylases for preparation of biologically active nucleosides. Search of optimal substrates.

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Keywords: (enzymatic synthesis of nucleosides; phosphorolysis nucleoside phosphorolysis; nucleoside phosphorylases; ribose 1-phoshate; 2-deoxyribose 1-phoshate)

Naturally occurring substances have always been the most abundant source of drugs. Nucleoside family in this regard is one of the most "fruitful" families of natural compounds: about a hundred of pharmaceutical substances were developed on their basis. The need to develop new nucleoside analogues and the wide range of their therapeutic activity has led to many efforts to optimize their synthesis.

Enzymatic reactions are widely used for preparation of biologically active nucleosides. Nucleoside phosphorylases (purine nucleoside phosphorylase EC 2.4.2.1, uridine phosphorylase EC 2.4.2.3, thymidine phosphorylase EC 2.4.2.4) catalyze the reversible phosphorolysis ribo- or deoxyribonucleosides to the corresponding heterocyclic bases and ribose-1-phosphate or deoxyribose-1-phosphate. Transfer of the carbohydrate moiety from nucleoside to heterocyclic base in the presence of this type of enzymes is called transglycosylation. This regio- and stereoselective process is a combination of two coupled equilibrium reactions of phosphorolysis of nucleosides (Nuc).

Phosphorolysis step: Nuc1 + P_i ↔ Sug-P + Base1;

Synthesis step: Base2 + Sug-P ↔ P_i + Nuc2.

The transglycosylation reaction was proven to be an efficient way for the nucleoside synthesis and is widely used in practice. We have compared the different substrates in this reaction and found that the highest yield of product can be achieved when 7-methyl-(2-deoxy)guanosine (7MeGuo or 7MedGuo) or α-*D*-(2-deoxy)ribose-1-phosphate (Rib-1-P or dRib-1-P) are used as substrates. We have developed useful protocols for preparation of these derivatives. In conclusion, we have developed an optimal strategy for the preparation of pyrimidine and purine ribo- and 2-deoxyribonucleosides using nucleoside phosphorylases.

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Study of structure and function of bacterial formate dehydrogenase

Yurchenko Tatyana

Apical domain of thermophilic chaperone as stabilizing fusion partner

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Keywords: protein stabilization, hydrophobic proteins, chaperones, fusion partners, GroEL.

The expression of recombinant enzymes is the key to a variety biotechnologically-derived products. The main obstacles in recombinant enzymes production are poor solubility and renaturation issues. There are some methods to maintain protein solubility and one of the major methods is fusion partners usage. Fusion partners usually are small soluble proteins with high expression rates. The most prominent fusion partner examples are SUMO, MBP and GST proteins. Moreover, MBP and GST proteins support their own affinity mode of protein purification. Fusion partners are conventional as scientific tool; however, they are few - if there are - large-scale production based on fusion tags technology.

We have applied a new approach to fusion partner engineering and developed a chaperone-based system named GrAD (stands for GroEL Apical Domain). GrAD is the substrate-binding domain of *Thermus thermophilus* HSP60 protein. It carries some hydrophobic regions among highly hydrophilic surface. These regions allow GrAD to bind excessively hydrophobic parts of target protein and prevent their aggregation.

The key features of that system are: outstanding stability, N-term/C-term and in-sequence target protein emplacement, thermal denaturation enrichment step in purification flow. As well as other fusion proteins, GrAD system provides high expression rates in *E. coli* expression system and significantly increases target protein content in soluble fraction of cell lysate.

It is well known that any fusion partner is individually compatible with a target protein. Some of these limitations could be overcome by proper linker design, yet the rational approach to fusion-based technology involves fusion partners screening to ensure the best performance. So, yet another effective and distinct fusion partner is a valuable option for troublesome proteins recombinant production.

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Linoleic acid obtaining from safflower oil by lipases from different sourses

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The unique specificity of the lipase, the high reaction rate, the possibility of carrying out the process under “mild” conditions, the absence of side products - all these factors make lipase attractive for use.

Linoleic acid (cis, cis-octadiene-9-12-one acid) is widely used as a drug in the treatment and prevention of many serious diseases (atherosclerosis, coronary disease and others.) The Sodium chloride, calcium ions, bile salts are important activators of lipolysis. Optimization of such conditions as salt concentrations as well as pH and temperature, had been carried out.

Lipases from microorganisms are more readily available than lipases from other sources, and improved genetic engineering methods increase the amount of lipases produced from recombinant bacteria and yeast. Hydrolysis of safflower oil by lipases *Alkaligenes, Burkholderia, Candida Cylindracea, Candida Rugosa* under optimal conditions (concentration of salts and surfactants, pH and temperature) had been performed.It was shown for all lipases under study, that in the range of surfactant concentrations below its CMC surfactant acts as an activator.

Development of the phospholipase A2 recombinant enzyme technology for the food industry

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Keywords: Phospholipase, food industry, enzyme activity, strain producer.

This work was carried out within the framework of the Federal target program "Research and development in priority areas of scientific and technological complex of Russia for 2014–2020" to ensure the conditions of the Agreement № 14.607.21.0207 on the provision of a subsidy concluded by the Ministry of Education and Science of the Russian Federation and the Federal State Institution «Federal Research Centre «Fundamentals of Biotechnology» of the Russian Academy of Sciences». It deals with the creation of a unified technological platform for the development and production of recombinant enzymes for the food industry, in particular, phospholipase A2 and chymosin, using the methanotrophic yeast *P. pastoris* as the producer.

During the work, strain producer of the phospholipase A2 enzyme was designed. Productivity of strain obtained not less than 50 mg of protein/l of medium (activity more than 500 U/ml). To obtain the producer, the following works were carried out:

- the codon optimization of the A2 phospholipase gene from *Streptomyces violaceoruber* for production in *P. pastoris*;
- the choice of vector and the signal sequence to obtain the maximum expression level;
- optimized cultivation conditions, such as the medium composition, temperature and time.

Recombinant enzyme production includes the concentration and purification stages. For this purpose, the following methods have been developed and optimized:

- concentration and removal of large impurities by ultrafiltration on TAMI ceramic filters with a pore size of 150 kDa and 10 kDa;
- removal of small impurities comparable to the size of the phospholipase A2 protein by cation exchange, anion exchange and hydrophobic chromatography.

Each stage was optimized as to minimize the loss of the target product. The final product had an activity of more than 10,000 U/ml and purity of 70-80 %.

A general technology for the production of the phospholipase A2 recombinant enzyme for the food industry has been developed. All stages are suitable for scaling. The technical requirements necessary and sufficient for the registration of the enzyme for the food industry in the Russian Federation have been drawn up, and samples complying with these requirements have been obtained.

Enhancement of ethanol and sensorial compounds production from cane molasses by manganese ferrite and co-culture use

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The present study focused on obtaining distillates from cane molasses from the Tamaulipas region of Mexico. For this, initially its characterization was done before being applied in alcoholic fermentation. Different tests were carried out to evaluate the effect of manganese ferrite (nanostructured magnetic system) and presence of co-culture of *Saccharomyces cerevisiae* 150 and *Kluveromyces marxianus* on the production of ethanol and sensorial compounds. Quantification was performed by means gas chromatography.

The cane molasses of the Tamaulipas region is characterized by several similarities when compared to that of Cuba, highlighting, however, a higher level of reducing sugars and a lower nitrogen content. The use of co-culture and chitosan-coated magnetic nanoparticles leads to obtaining higher levels of ethanol in shorter fermentation times. The levels of volatile compounds detected in distillates are within the range allowed by the Mexican norm.

Yeast immobilization by adsorption on ferrite nanoparticles was demonstrated. Immobilized cells were easily removed from fermentation media under external magnetic field that simplified biomass re-use in different fermentation cycles. Use of re-used biomass led to decrease in fermentation time and increase ethanol level. The best results were observed in case of mixed culture immobilized in magnetic system.

The use of yeasts immobilized in manganese ferrite magnetic nanoparticles allows to obtain distillates that can be applied in the production of rum with improved sensorial properties.

Keywords: Alcoholic fermentation, congeners, nanoparticles, molasses cane.

Novel enzymes for biosynthesis and biotransformation of adipic acid

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At present, the most thermodynamically feasible pathway for adipic acid production is shikimate pathway. Adipic acid is one of the most important aliphatic dicarboxylic acids, which is used for the synthesis of nylon-6-6 and also can be utilized for further biotransformation to 1,6-hexendiol and hexamethelendiamine. Here we focused on several important groups of enzymes participating in the shikimate pathway and also targeting subsequent biotransformation of adipic acid to other industrially valuable compounds (Khusnutdinova et al., 2017). Enzymes we are focused on are protocatechuic acid (PCA) decarboxylase (UbiD family of proteins) which requires prFMN as a cofactor (Wang et al., 2018), that is produced by flavin prenyltransferase (UbiX family of proteins); and 2-enoate reductases. UbiD decarboxylases are important industrial enzymes that are used for vanillin, vine and paper production (White et al., 2015**).** Their activity and application is hindered by prFMN cofactor biosynthesis. We screened more than 20 different UbiX enzymes and identified several FMN prenyltransferases suitable for aerobic activation of UbiD decarboxylases.
Final step of adipic acid synthetic pathway relies on muconic acid double bonds hydrogenation, that previously was performed by non-enzymatic catalysts. Recently, we demonstrated the first enzymatic hydrogenation of muconic acid and 2-hexenedioic acid to adipic acid using bacterial 2-enoate reductases (ERs) (Joo et al., 2017). Based on the structural model of the *Bacillus coagulans* ER (ERBC) over 40 ERBC residues were selected for site-directed mutagenesis, and 29 mutant ERBC proteins were purified and used for *in vitro* activity assay. Presently, based on phylogenetic analysis of Enoate reductase we identified oxygen stable ER applicable for *in vivo* catalysis.
Subsequent biotransformation of adipic acid to 1,6-hexendiol, 6-aminocaproic acid and hexamethelendiamine has been performed using coupled *in vitro* assay with carboxylate reductases, aldehyde reductases and amino transferases. We achieved ~ 5-10% *in vitro* transformation efficiency to hexamethylenediamine, ~ 40% *in vitro* transformation to 6-aminocaproic acid, over 95% *in vitro* and *in vivo* transformation efficiency to 1.6-hexendiol.

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Fundamental technologies of biocatalytic preparation of acrylic monomers

Lavrov Konstantin

Penicillin acylase in chemoenzymatic synthesis of peptidomimetics

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Keywords: penicillin acylase, peptidomimetics, chemoenzymatic synthesis, chiral thiols

Peptidomimetics are considered as perspective biologically active compounds possessing diverse attractive properties [1]. Chemoenzymatic synthesis of peptidomimetics is one of the most promising directions of fine organic synthesis thus combining the stereo-, regio- and chemoselectivity of biocatalysis and the efficacy of chemical transformations [2,3]. Among successful applications of different enzymes in this area penicillin acylases were shown to be effective for protection and deprotection of amino groups [4-7]. In this work we have studied a possibility of using penicillin acylases to introduce halogen-substituted acyl groups for protection of amino groups in a wide range of compounds under mild reaction conditions in an aqueous medium. Subsequent replacement of chemically active halogen then opens up new possibilities for the design of a wide range of peptidomimetics bearing different functional groups. Acyl transfer to amino acids catalyzed by penicillin acylase from *Alcaligenes faecalis* using chloroacetamide as an acyl donor and subsequent chemical substitution of halogen for H-, SH, SO_3-, as well as thiourea derivatives was investigated. The use of halogen-substituted acyl donors allows to expand catalytic potential of penicillin acylases in organic synthesis and produce a range of new peptidomimetics. Preparative synthesis of N-sulfoacetyl-L-phenylalanine and a new chiral thiol N-thioacetyl-L-phenylalanine was performed with high overall yield.

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Search for biocatalysts of polydepsipeptide synthesis

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Keywords: biodegradable polymers, polydepsipeptides, polyesteramides, lipase, enzymatic synthesis

2,5-Diketomorpholine derivatives and esters of N-oxyacylated amino acids could be promising monomers for the enzymatic synthesis of biodegradable polyesteramides (polydepsipeptides). Polydepsipeptide-based bioresorbable polymers can possess different functional properties and be used in controlled drug release systems, tissue engineering scaffolds and as shape-memory materials [1]. Polyesteramides as well as polyesters are prepared by consequtive ester bond-formation reactions employing two major approaches: ring-opening polymerization of cyclic monomers such as 2,5-diketomorpholines and polymerization via condensation of a carboxylic acid (or its ester) and an alcohol group of bifunctional monomers [2]. Lipases are most commonly used as biocatalysts to prepare polyesteramides by ring-opening polymerization of cyclic monomers [3].

Earlier we have reported a chemoenzymatic procedure of the stereoselective synthesis of 2,5-diketomorpholine derivatives and esters of N-oxyacylated amino acids [4]. In this work, we have investigated the possibility to use the earlier synthesized compounds as monomers for lipase-catalyzed polymerization applying molecular modeling as well as experimental studies. Molecular docking has shown that lipase B from *Candida antarctica* is capable of forming reactive enzyme-substrate complexes with both 2,5-diketomorpholine derivatives and esters of N-oxyacylated amino acids. The calculated binding energies were −8 ÷ −6 kcal / mol. Different lipase preparations have been also tested experimentally for their ability to convert these substrates. The activity of each enzyme preparation was studied experimentally using N-(R)-mandelyl-(S)-phenylalanine methyl ester and (3S,6S)-3-benzyl-6-phenylmorpholine-2,5-dione as model substrates in the biphasic system toluene-aqueous phosphate buffer. It was shown that lipases can catalyze the transformation of previously unexplored substrates. The methodology to track biocatalytic polymer synthesis based on the analysis of the molecular mass distribution of the reaction products using exclusion chromatography has been developed and optimized.

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Immobilization and application of purified fungal chitinase

Shora Hamed

Biocatalysts. Hydrolases Production

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Keywords: hydrolases, production, scaling-up

The need to reduce waste generation and the use of toxic materials for waste disposal has led to a growing interest in the development of sustainable bioprocesses using enzymes. Hydrolases can be applied in different fields such as biofuels, pharmaceutical, detergent, oleochemical, organic synthesis and food industries. Many hydrolases such as, lipases, proteases and glycosyl hydrolases are extracellular, and to make possible its use economically, it is necessary to optimize yields through optimization of culture conditions and/or genetic manipulation to increase the microbial enzyme activities.

The objective of this work was to optimize hydrolases production, and the design of bioprocess scale-up. The culture medium to obtain high enzyme activities by different strains belonging to *Firmicutes* phylum was optimized by means of statistical-based designs, followed by a partial purification to assess the enzyme preparations properties.

A Box Behnken design-based statistical analysis indicated that the variables that most influenced the lipase and protease production were peptone and sucrose concentration. Increments on lipase production up to 33 U/g at 5 g/L of sucrose were observed, while further increments on the carbon source concentration reduced the enzyme production. The optimized medium (sucrose, 5g/L; peptone, 7.5 g/L; CaCl2, 0.05 M) produced 35.1 U/mL and 4.60 U/ml for lipase and protease respectively. Both, protease and lipase, were used to enrich refined fish oils in polyunsaturated fatty acids (ω-3 PUFA); as a result, an increment of 1.2-fold of DHA (docosahexaenoic acid) content was obtained.

The examination of the culture media components influencing bacterial growth and endoglucanase production demonstrated that mono and disaccharides are useful substrates for enzyme production. Moreover, their combination with carboxymethyl-cellulose (CMC) showed a synergistic effect on enzyme production. Consequently, by using a peptone-based medium amended with sucrose and CMC, a cellulolytic cocktail was efficiently produced in a 1-L stirred tank reactor on batch operation mode, reaching a maximum of 3.12 IU/mL. It is important to point out that the use of simple sugars favors both operative culture conditions and downstream processing.

Finally, raw and alkali-pretreated sugarcane bagasse were the carbon sources that better promoted xylanase production. The xylanases obtained were further utilized for: (i) a cocktail formulation supplementing commercial enzymes that improved the glucose releasement (~38 %) from sugarcane pretreated material for second generation ethanol production. (ii) generation of arabino-xylooligosaccharides, potential prebiotics, from the pretreated sugarcane bagasse and wheat bran.

Scaling-up these bioprocesses to evaluate the technical feasibility and the commercial viability is now being performed in a pilot plant with a 150 L Fermentor. Scale parameters were selected for each of the enzymes.

Novel Recombinant Phytase Preparation obtained by *Penicillium* sp.

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Keywords: phytase, phytate, phosphorus, animal feed additive, *Penicillium* sp.

Phytases (EC 3.1.3.26) are the phosphatases, that hydrolyzed phytate into mio-inositol and inorganic phosphate, which are easily digests by the poultry, pigs and other monogastric animals. Without phytase up to 70% of phosphorus in feedstuffs of plant origin is unavailable to the animal and is secreted in manure. Microbial phytases provides the user a broad range of advantages and are very promising for their commercial exploration, especially compared to phytases produced by plants or animals.

A heterologous phytase gene was cloned to the *Penicillium* sp. fungal host and a highly productive strain of phytase was obtained. As a final product a dry enzyme preparations with the activity over 50000 phytase units per g and with the phytase content higher than 40% from the total protein was obtained. Optimal pH for purified phytase is 4.5-5.5, optimal temperature – 45-60ºC. Phytase showed sufficient stability: it remained 80 and 60% of its original activity after incubation at 40 and 50ºC respectively for 3 hours. With its acid pH optimum and good temperature stability, as well as with high *Penicillium* sp. recombinant fungal host productivity this new phytase appeared to be a perfect candidate for animal feed application.

Biocataltic synthesis of the conducting copolymer of aniline and 2-aminophenethyl alcohol using a laccase-mediator system

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Keywords: biocatalytic synthesis, conducting polymers, laccase-mediator system, matrix polymerization, copolymer of aniline and 2-aminophenethyl alcohol

Poly(aniline-co-o-aminophenethyl alcohol)s were synthesized on poly(2-acrylamido-2-methyl-propanesulfonic acid) matrix (PAMPS) using a laccase-mediator system (LMS). Highly redox-potential laccase from the fungus *Trametes hirsuta* was served as a catalyst for polymerization and hydroxybenzotriazole (HBT) was used as a mediator. As a result of one-step synthesis, the conductive copolymer was obtained, the primary alcohol groups of which were oxidized to aldehyde groups. The formation of carbonyl groups in the copolymer chain was confirmed by a silver mirror test and FTIR studies. Peaks at 1740 and 1207-1220 cm-1 were observed on the FTIR spectrum of the copolymer, which can be attributed to tensile and deformation vibrations of the carbonyl group.

For the modification of the copolymer laccase substrate L-dioxyphenylalanine (DOPA) was used. Since the optimum pH of *T. hirsuta* laccase activity is 4.5 and Schiff bases are unstable in acidic medium the forming copolymer/DOPA conjugate was reduced with sodium borohydride. As a result of laccase-catalyzed oxidation of DOPA the product with an absorption maximum in the 570 nm area was formed in the conjugate.

Thus, the possibility of using laccase-mediator systems for synthesis of the conducting copolymer of aniline and 2-aminophenethyl alcohol with reactive aldehyde groups was demonstrated. Aldehyde groups in the copolymer allow to bind various amino compounds in order to obtain conducting composites that can be useful for biosensor devices, neural interfaces, etc.

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Biocatalytic synthesis of conducting polyaniline for development of effective coatings against corrosion and static electricity

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Keywords: enzymatic polymerization, laccase, conducting polymers, polyaniline, dodecylbenzenesulfonate matrix, antistatic and anticorrosion agents

The problem of protecting various surfaces from corrosion and static electricity is very relevant. Promising for these purposes is the use of conducting polyaniline (PANI). An enzymatic synthesis of conducting polyaniline (PANI) was performed in micellar solutions of the anionic surfactant sodium dodecylbenzenesulfonate (DBS). Laccase isolated from the culture fluid of *Trametes hirsuta* fungi served as a catalyst for the oxidative polymerization of aniline. The synthesis conditions have been optimized. The resulting PANI/DBS complexes were characterized by various physicochemical methods. The electrical conductivity of PANI measured by the standard four-point method was 0.7 mMs/cm.

Anticorrosion and antistatic properties of the synthesized PANI/DBS complexes were tested. Electrochemical corrosion was modeled using the process of dissolving copper in an acidic environment under the action of an external electric potential. For this purpose the method of linear anodic voltammetry was applied. High purity copper (99.9%) was used as the electrode material, the measurement medium serving 0.1 M HCl.

It was shown that PANI/DBS films deposited from toluene on the copper surface exhibited high adhesion to the metal surface and served as a protective anodic coating passivating and stabilizing the copper surface. The antistatic properties of PANI were estimated from the electrostatic discharge rate. The half-lives of positive and negative charges on the surface of cotton fabric treated with PANI/DBS complexes were 3.8 and 5.8 s, respectively, which is significantly less than the time of electrostatic discharge for control samples treated with DBS solution (154 and 114 s).

Thus, conducting PANI obtained by enzymatic polymerization of aniline on a DBS matrix is a very effective antistatic and anticorrosion agent. This makes it promising for use as a protective coating.

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Biocatalytic synthesis of conducting poly(3,4-ethylenedioxythiophene) using "soft" natural template DNA

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Keywords: enzymatic synthesis, fungal laccase, conducting polymers, 3,4-ethylendioxytiophene, DNA, matrix polymerization

Enzymatic oxidative polymerization of 3,4-ethylendioxytiophene (EDOT) was carried out using "soft" natural polyelectrolyte DNA as a template. As known, phosphate groups of DNA bind other molecules via electrostatic interactions or/and hydrogen bonding, thus DNA can be served as a dopant of the main chain of PEDOT. A commercially available sodium deoxyribonucleate (Derinat) containing DNA molecules from 50 to 900 base pairs was used. Conductive biocompatible interpolymer complex PEDOT/DNA was obtained by enzymatic EDOT polymerization with atmospheric oxygen as a terminal oxidant. High redox potential fungal laccase *Trametes hirsuta* was used as a biocatalyst of the reaction. The resulting biocomposite formed a stable aqueous-dispersible system. PEDOT/DNA conductivity measured by standard four-point method was of ~0,15 mS/cm. Some physicochemical characteristics of obtained complex and its morphology have been investigated. PEDOT/DNA complex may be used in various biodevices, for example, biosensors, flexible biocompatible neurointerfaces, implantable supercapacitors and others.

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The application of physico-chemical and biological methods to suppress the processes of gas generation in grounds

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Keywords: biogas generation rate, gas-generating grounds, gas potential, anaerobic processes, calcium hypochlorite.

As a result of man-made activities, gas-generating grounds (GG) have been accumulating for decades, containing impurities of decomposing organic substances and capable of producing biogas, consisting of combustible components - hydrogen and methane. Such grounds include soils enriched with organic matter, landfills for municipal solid waste, soils of irrigation fields and sewage sludges.

As the territories containing GG are often used for housing construction, the development of methods for decontamination of grounds capable of generating toxic and explosive biogas is highly relevant. The most promising method of GG neutralization is a conversion in the properties of grounds, aimed primarily at suppressing their gas functions by various physical (mechanical, hydrodynamic, aerodynamic, thermal effects on GG), chemical (hydrolysis, chemical precipitation and oxidation of pollution) and biological (activation of microflora and bioaugmentation) methods in situ.

Studies were conducted on simulation of suppressing gas formation in the course of deactivation of gas-generating grounds (GG) by their chemical and biological treatment in anaerobic conditions. The chemical and gas-geochemical characteristics of GGs sampled from wells of different depths drilled within the area of a construction site (Moscow, NEAD) were examined. Simulation of an anaerobic gas generation process showed that the gas generation rate under conditions of natural occurrence (8–10°C) within the boundaries of identified anomalous zones would be about 215 l/(t·year) in the center and 157 l/(t·year) in the north of the site. The kinetics of organic substances degradation revealed the rate constant for gas-forming substrates degradation of 0.013 $year^{-1}$ for the central zone, and 0.009 $year^{-1}$ for the northern zone. According to the calculations, the main gas generation phase would be completed within 55 years in the central zone, and within 79 years in the northern one, while methane volume content of in the gas mixture would be 9–12%.

Simulation of chemical suppressing residual gas generation showed that 10-fold reduction of gas generation intensity could be achieved by adding 45% calcium hypochlorite solution at a rate of 1% of the overall soil volume into the GG-massive in the central anomalous zone. The gas generation in grounds after their chemical treatment would vary in the range from 1.6 to 35 l/(t·year), i.e. the process of gas generation in fill-up grounds would be suppressed almost completely.

Simulation of biological process of gas generation suppression in the northern zone, caused by the presence of oil pollution, made it possible to predict that applying the certified oil degrader Rhoder at a dose of $1 \cdot 10^9$ CFU/ml would significantly raise the rate of hydrocarbon degradation up to 1.02 $year^{-1}$ and at the same time enhanced reducing the half-life decay period of hydrocarbons from 79 years to 0.68 years.

Heterogeneous and homogeneous biocatalysts for the biodegradation of nitriles and acrylamide

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Keywords: nitrile hydrolyzing bacteria, amidase, bioaugmentation, biofilter

Carboxylic acid nitriles (acrylonitrile, acetonitrile) and some amides (acrylamide) are toxic compounds released into the environment with industrial effluents. Nitrile hydrolyzing bacteria containing nitrilase, nitrile hydratase and amidase can serve as biocatalysts for the degradation of toxic compounds. Biomass with such enzymatic activity can be used in a homogeneous form for inoculation of polluted media, or in a heterogeneous form, for example, in the form of biofilms grown on a carrier that fills the biofilter.

The aim of the work was to study the biodegradation of acrylamide in river sludge when inoculating biomass of bacteria with amidase activity and biotransformation of acrylo- and acetonitrile in biofilter with biofilms grown on carbon and mineral carriers.

Biomass of *Alcaligenes faecalis* 2 and *Acinetobacter guillouiae* 11h with amidase activity was added to the suspension of river sludge and the degradation of acrylamide was studied. It was determined that the initial rate of degradation was the highest at 30°C when inoculated with *A. guillouiae* 11h biomass and amounted to 2.6–3 mg/h/g of dry sludge, while at 5°C – 0.7–1.3 mg/h/g. Without inoculation with the biomass of amidase-containing strains, the suspension of silts transformed acrylamide only at a temperature of 30°C. The survival rate of the foreign microbiota was studied by the method of metagenomic sequencing and by transformation of acrylamide with suspensions of silts after storage for 3 and 6 months at temperatures of 5 and 22ºC. *Alcaligenes* sp. was not detected in river muds without inoculation. However, after 6 months storage at 22ºC suspensions of silts inoculated with *Alcaligenes* sp. bacteria, the content of this genus was 1.6–9% of the microbiome as a whole.

The transformation of acetonitrile and acrylonitrile in a submerged packed-bed biofilm reactor was carried out by *Rhodococcus ruber* gt 1 and *A. faecalis* 2 with high nitrile hydratase and amidase activity, respectively. Biofilms were grown on mineral and carbon carriers. *R. ruber* gt 1 cells with a hydrophobic cell surface form a more massive biofilm on carbon carriers, more hydrophilic *A. faecalis* 2 – on mineral carriers. We used 2 types of biofilters: 1) based on mixed biofilms on mineral and carbon carriers; 2) on the basis of two types of carriers in equal proportion with the grown monofilms. During the transformation of acetonitrile in a biofilter based on mixed biofilms on a carbon carrier, accumulation of acetic acid in the reactor was noted; on mineral carrier after 600–800 hours of adaptation, complete mineralization was observed. A biofilter based on mixed carriers with biofilms worked for 3000 hours, the nitrile was completely utilized, with a slight accumulation of acetic acid.

Conclusion: 1). It was shown that the degradation of 7 g/l of acrylamide added to the suspension of river sludge at 5°C can be carried out only when the biomass is inoculated with microorganisms selected in the direction of increasing the amidase activity. 2). A good survival rate of amidase strains of *A. faecalis* 2 and *A. guillouiae* 11h in river sludge was shown. 3). Biofilters based on mono- and mixed biofilms of nitrile-hydrolyzing bacteria are promising for removing nitriles from water.

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New Soil Strains-Destructors of Phenol

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Keywords: phenolic, phenolic compounds, degradation, MALDI-tof mass-spectra

Today, contamination with organic pollutants (phenols, pesticides, etc.) is in third place in terms of the prevalence after radioactive contamination and pollution with crude oil and products of its processing. According to various sources, the concentration of phenol in wastewater can get to 0.5 g/l [1, 2] or to 30 g/l [http://enviropark.ru/course/info.php?id=49]. Removal of the most pollutants from the contaminated environment using ordinary physical and chemical methods is an expensive and non-ecological process. On the other hand, phenol and its derivatives are decomposed by a variety of microorganisms into non-toxical metabolites. The search for highly effective and safe for living methods of purifying biotopes from technogenic pollution is an important problem of the present time.

The aim of this research is to identify new soil microorganisms that can decompose phenol in high concentration.

More than 80 strains of microorganisms isolated from various contaminated and uncontaminated soils (Saratov Oil Refinery, contaminated and uncontaminated soils of the city of Pushchino) were used in the work. The ability of bacteria to decompose phenol was tested by cultivating the selected strains in a mineral medium with phenol (0.1, 0.3, 0.5 and 1.0 g/l) with a stepwise cultivation of the cultures into the medium with an increase in substrate concentration. Thus, out of 80 isolated strains, 11 were selected for their ability to degradation phenol at a concentration of at least 0.5 g/l.

Part of the cultures was identified. Analysis of the 16S rRNA gene showed that the selected strains belong to the different genera: *Rhodococcus*, *Pseudomonas* and *Stenotrophomonas*. The characteristics of the cytological and biochemical properties of the selected strains are given. It is shown that the cultivation of bacteria in a liquid medium with phenol leads to a decrease in the cell size. The coccoid stage is characteristic of the rhodococcus, the sticks were observed only on the first day of growth.

The results of the strains identification by analysis of the 16S rRNA gene are in accordance with the data on the biochemical characterization of the bacteria, carried out using API 32E and 50CH tests (“bioMerieux”, France). The MALDI-tof mass-spectra of some selected strains grown on benzoate, phenol and rich Luria-Bertani (LB) agar medium were obtained. It was found that protein profiles differed significantly among representatives of the different taxonomic groups.

Further work will be aimed at determining features of the phenol destruction process by selected strains.

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Association of polymorphic markers of *TP53* and *CDKN1A* genes with the progression free survival of ovarian cancer patients after platinum-based chemotherapy

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Keywords: ovarian cancer, polymorphic marker, cell cycle control

Ovarian cancer is a socially important disease. In oncogynecology it occupies the 2nd place in incidence and the 1st place in mortality. One of the most effective first-line chemotherapy regimens for ovarian cancer is based on platinum derivatives in combination with paclitaxel. However the platinum resistance of tumors remains a key question in the treatment of ovarian cancer. Under the action of platinum drugs double-stranded breaks generate in DNA molecule. It leads to cell death when it passes through the cell cycle. The key genes controlling this process are *TP53* and *CDKN1A*.

The aim of this work was to study the polymorphic markers of *TP53* and *CDKN1A* genes and their relationship with the progression-free survival time (PFS), which is a surrogate clinical marker of sensitivity of ovarian cancer to platinum drugs. The study was included 26 patients with advanced ovarian cancer (stage II-IV). The samples of tumor were selected from patients before the start of chemotherapy, during the primary cytoreductive surgery. After surgery, all patients received standard chemotherapy using paclitaxel and platinum drugs. DNA was isolated from tumor tissue samples using the Diatom DNA Prep 400 kit (Isogen, Russia). The polymorphic markers *Arg72Pro* of the *TP53* gene and *Ser31Arg* of the *CDKN1A* gene was analyzed by PCR-RFLP and real-time PCR melting curves analysis as reference. The results of markers analysis were compared with the duration of PFS.

A trend for shorter duration of PFS in the presence of the *Pro* allele (in 1.4 times) of the *Arg72Pro TP53* marker was revealed (the median PFS was 11.8 months in patients with the *Pro* allele compared to 17.0 months in the subgroup of patients with the absence of the *Pro* allele). In the subgroup of patients with optimal cytoreductive sergery, the effect of the carriage of the minor *Arg* allele of the *Ser31Arg* marker of the *CDKN1A* gene on the duration of PFS was revealed. With the presence of the minor *Arg* allele, a statistically significant decrease of the duration of PFS after platinum-containing chemotherapy was observed (the median PFS in the absence of the *Arg* allele was 19.08 months, in the presence of the *Arg* allele – 12.82 months, $p = 0.035$). As a result, the advisability of further studying of used molecular genetic factors on a representative group of patients with ovarian cancer was revealed.

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Inactivating and stabilizing effects of glycosaminoglycan ligands during molecular docking of hyaluronidase

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Role of glycation and glycolysis in the induction of neurodegenerative amyloid diseases

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Cyclic hydroxamic acids as effective inhibitors of histone deacetylases

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Key words: hydroxamic acids, inhibitors of histone deacetylases, antioxidants.

Cancers are an acute problem of modern medicine. One of the main methods of treating oncological pathological conditions has been and remains now - the method of chemotherapy. Therefore, the search for substances of potentially effective antitumor agents among new directionally synthesized compounds is currently an urgent task.

Such compounds may include hydroxamic acids, inhibitors of histone deacetylases (HDACs), enzymes that play an important role in the development of carcinogenesis. In this work, several new target compounds were synthesized, containing in their base a fragment of hydroxamic acid and additional quinazolin-4 (3H) -one or dihydroquinazolin-4 (1H) -one modules. A study of their biological activity was carried out: the antioxidant status was determined according to the degree of influence on lipid peroxidation of rat brain homogenate, the antiradical activity of the DPPH test and the ability to chelate iron ions were detected. For hit compounds, inhibitory activity against HDAC1 enzyme and cytotoxicity on cells of various tumor lines (A549, MCF7 and others) was tested.

From the studied hydroxamic acids, a group of substances with high antioxidant, antiradical and iron chelating activity was isolated. For most of them, inhibitory activity against HDAC1, which correlates with the percentage of Fe (II) chelating ability, has been shown. The most effective hydroxamic acids in previous tests have a cytotoxic effect on A549 and MCF7 tumor cell lines. Thus, hydroxamic acids with quinazolin-4 (3H) -one or dihydroquinazolin-4 (1H) -one fragments can be considered as the basis for creating potential chemotherapeutic agents for treating cancer.

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Novel acetylcholinesterase inhibitors based on 6-methyluracil moiety for Alzheimer's disease treatment

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Keywords: Alzheimer disease, acetylcholinesterase, β-amyloid.

Reduction of β-amyloid (Aβ) production and increasing clearance of Aβ pathogenic forms are key targets in the development of oncoming therapeutic agents for Alzheimer disease (AD) treatment. Currently the primary therapeutic treatment for the AD is a cholinergic replacement strategy by acetylcholinesterase (AChE) inhibitors. Unfortunately, this strategy is unable to slow down neurodegeneration. It was shown that AChE itself promotes the formation of Aβ fibrils and plaques. This property of AChE resulted from interaction between Aβ and the peripheral anionic site of the enzyme (PAS).Thus bifunctional inhibitors that simultaneously interact with both the catalytic and PAS of AChE may act as disease-modifying agents with multiple functions, simultaneously improving cognition and slowing down the rate of Aβ-induced neural degeneration. Nevertheless, the assortment of AChE PAS ligands is still extremely limited.

Compound 2b based on 1,3-bis[ω-(substituted benzylethylamino)alkyl]uracil derivatives is mixed-type reversible inhibitor of cholinesterase with the half maximal inhibitory concentration (IC_{50}) for AChE=7,28±0,42x10^{-9}M. It show selectivity for human AChE 13736 fold higher than that for human butytylcholinesterase.

Compound 2b was found to improve working memory on scopolamine mouse model of AD in T-maze. Scopolamine injected mice showed significant impairment of spatial memory. This memory deficit was rescued to some extent by compound 2b (1-10 mg/kg) or donepezil treatment (1 mg/kg). The most effective rescue of memory impairment was found in case of compound 2b treatment with dose 5 mg/kg. However, there was no significant differences found between efficiencies of 2b (5 mg/kg) and Donepezil (1 mg/kg) treatment in rescue of spatial memory deficit in scopolamine injected mice ($P>0.05$).

Thus AChE inhibitors based on 1,3-bis[ω-(substituted benzylethylamino)alkyl]uracil derivatives exhibited outstanding selectivity against AChE and produced therapeutic effect on spatial memory deficit.

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A trifluoroketone, slow-binding inhibitor of human acetylcholinesterase, acting as a transition state analog of acetylcholine

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Keywords: Acetylcholinesterase, slow binding inhibitors, Alzheimer's disease.

Transition state analogues are effective slow binding inhibitors (SBI) of acetylcholinesterase (AChE) [1] and can show prolonged pharmacological efficacy with minimal unwanted side effects. Kinetic and MM studies of a fluorinated acetophenone derivative, 1-(3-tert-butylphenyl)-2,2,2-trifluoroethanone (TFK), was performed on human AChE.

Kinetic studies of human recombinant AChE inhibition by TFK were performed using Ellman's method in 0.1 M sodium phosphate buffer (pH 8.0) and acetylthiocholine as the substrate. Assays were initiated by addition of the enzyme. Absorbance change was recorded for 60 min. Molecular docking was performed with AutoDock 4.2.6, MD simulations with Namd 2.12 and QM/MM with NwChem 6.6 software.

Fast reversible inhibition of AChE by TFK is of competitive type with Ki =4.79 nM. However, steady state of inhibition is reached slowly, and kinetic analysis showed that TFK is a SBI of type B with Ki* = 0.52 nM. This type SBI results from "isomerization" of the initial enzyme-inhibitor complex, in fact a slow induced-fit step, which leads to an increase in binding affinity of one order of magnitude. TFK has a long residence time, τ=21.6 min, on AChE.

Molecular docking and MD simulations depicted the different binding steps of TFK. It was shown that TFK binds first to the AChE peripheral anionic site. Then, the subsequent slow induced fit step corresponds to expansion of the gorge, allowing tight adjustment into the catalytic active site. Modeling of the reaction between TFK and AChE active site by QM/MM method shows that this "isomerization" step of enzyme-inhibitor complex leads to a complex similar to substrate tetrahedral intermediate, traditionally called in literature "transition state analog".

Thus, this SBI TFK capable of binding to human AChE with high affinity could be of interest in therapy of Alzheimer's disease (AD) neuroprotection. It is already subject of clinical investigations for neuroimaging of neurodegenerative diseases [2]. The related silyl compound, Zifrosilone, a slow tight binding inhibitor of type A with with τ=70 h and Ki=0.26 nM for rat brain AChE [3] was promising for symptomatic treatment of AD. However, human clinical trials were discontinued likely because of its too longer residence time (τ>50 h) on target. TFK is more suitable for further research as an effective and safer pharmacological drug.

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Association of molecular genetic markers of cell cycle control and DNA repair genes with progression free survival of ovarian cancer patients after platinum-based chemotherapy

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Keywords: ovarian cancer, platinum drugs, cell cycle control, DNA reparation, DNA methylation

The most important aim of the modern clinical oncology is the personalized treatment especially because of the high toxicity of chemotherapeutic drugs. The key drugs used in chemotherapy of ovarian cancer (OC) are platinum derivatives, which are both high efficacy and high toxicity. This makes relevant to search for sensitivity markers to this group of drugs.

The aim of this work was to study molecular genetic markers of DNA repair genes and the cell cycle control genes and their relationship with the progression-free survival time (PFS), which is a surrogate clinical marker of sensitivity of OC to platinum drugs. The study was included 31 patients with advanced OC (stage II-IV). The samples of tumor were selected from patients before the start of chemotherapy, during the primary cytoreductive surgery. All patients received standard chemotherapy using platinum drugs and paclitaxel after surgery. The polymorphic markers *Gln399Arg* of *XRCC1* gene, *Lys751Gln* of *ERCC2*, *Arg72Pro* of *TP53, T(-410)G* of *MDM2, Ser31Arg* of *CDKN1A*, and mutation *5382insC* of *BRCA1* were analyzed by PCR-RFLP and real-time PCR melting curves analysis as reference. Methylation of *BRCA1* and *TP53* promoter regions was studied in 19 pared probes of blood and cancer tissue using method of bisulfite conversion followed by methyl-specific real-time PCR. The results of analysis of markers were compared with the duration of PFS. A tendency towards a longer duration of PFS in the presence of *Gln* allele of *Gln399Arg XRCC1* ($p=0.07$) and a tendency towards a shorter duration of PFS in the presence of the *G* allele of the *T(-410)G MDM2* ($p=0.06$) was revealed. In the subgroup of patients with optimal cytoreductive sergery, a statistically significant decrease of the duration of PFS in the presence of the *Arg* allele of *Ser31Arg CDKN1A* was observed ($p=0.04$). A trend for shorter duration of PFS (median, M) in the presence of the *Pro* allele of the *Arg72Pro TP53* (M(*Pro*+)=11.8 months, M(*Pro*-)=17.0 months) and *Gln* of *Lys751Gln ERCC2* (M(*Gln*+)=14.1 and M(*Gln*-)=18.3 months) was found. Hypermethylation of *BRCA1* promoter in 3 samples of tissue was obtained. All these patients had no progression of disease and a trend to longer duration of PFS was obtained (18.0 compared to 15.1 months).

The obtained results suggest the advisability of further studying of these molecular genetic factors on a representative group of patients with OC.

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Bioluminescent indication of the temperature stress on prokaryotic cells

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Keywords: bioluminescence, firefly luciferase, ATP, thermal stability, metabolic activity, prokaryotes

Monitoring of cell viability under the stress is one of the promising areas of research. The purpose of this work was to study the effect of temperature on the viability of *E.coli* expressing the mutant firefly luciferases, by measuring the bioluminescence intensity of the endogenous luciferase and the content of intracellular ATP.

The object of the study were *E. coli* BL21 DE3 Codon plus cells transformed with the pETL7 plasmid carrying the firefly luciferase *Luciola mingrelica* genes: wild-type (WT), thermostable (TS), and green (GTS) mutants. Luciferase was expressed in soluble and active form, and its enzymatic activity was measured by bioluminescence intensity of cells without their destruction on the luminometer (LUM 1, Russia) using a luciferin-based substrate mixture.

It was shown that in the temperature range of 42-60 ºC there was a correlation between the enzyme activity of endogenous luciferase and the cell viability (CFU/ml, measured by culture method). Moreover, the activity of the enzyme isolated from the cells after their heating correlated with the activity of endogenous luciferase measured by bioluminescence intensity of intact cells. This indicated that the decrease in luciferase activity during heating occurs precisely due to the denaturation of the enzyme, and not due to a decrease in the concentration of the components necessary for the reaction of bioluminescence to take place.

The amount of intracellular ATP during the cell heating was determined by bioluminescent method for all *E. coli* cells producing luciferase mutants (WT, TS and GTS) and for non-transformed *E. coli* cells. For all samples, a sharp (3-5 fold) increase in ATP level was observed in the first 20 minutes of heating, followed by a gradual decrease in the signal. The rate of decline in ATP was significantly lower than the decrease in cell viability. It has been suggested that an increase in the rate of ATP synthesis at the initial stage of heating is associated with the activation of the protective functions of living cells under conditions of temperature stress, which requires serious energy expenditure.

A mass spectrometric study of sphingolipids, biomarkers of amyotrophic lateral sclerosis

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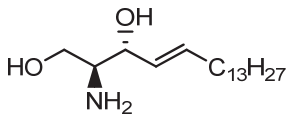
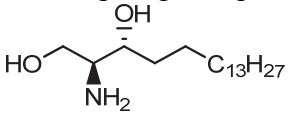
Keywords: HPLC-MS, MRM, sphingolipids, amyotrophic lateral sclerosis

Amyotrophic lateral sclerosis (ALS) is an incurable neurodegenerative disease leading to selective degeneration of motor neurons in spinal cord, motor cortex and brainstem. ALS is accompanied by lipid metabolism disorders. Sphingolipids are important signal transduction messengers that regulate cell proliferation, differentiation and apoptosis. Specifically, ceramides and sphingosines are proapoptotic, while sphingosine-1-phosphate protects cells from apoptosis.



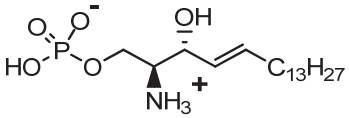
sphingosine

$MH^{+\cdot} \rightarrow (M\text{-}2H_2O)^{+\cdot}$
m/z 300 → m/z 264



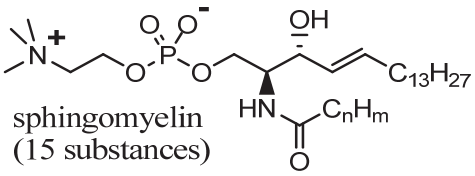
sphinganine

$MH^{+\cdot} \rightarrow (M\text{-}2H_2O)^{+\cdot}$
m/z 302 → m/z 266

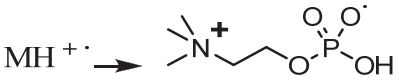


spingosine 1-phosphate

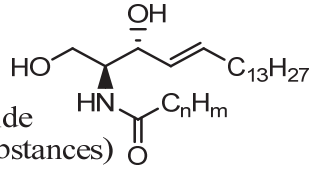
$MH^{+\cdot} \rightarrow (M\text{-}H_3PO_4\text{-}H_2O)^{+\cdot}$
m/z 381 → m/z 264



sphingomyelin
(15 substances)



m/z 184



ceramide
(15 substances)

$MH^{+\cdot} \rightarrow$ (MH-acyl-H_2O-OH)
m/z 264

$(MH\text{-}H_2O)^{+\cdot} \rightarrow$ m/z 264

We studied changes in the concentrations of each sphingolipid in every group (see the scheme) in hippocampus, cerebellum, brain cortex and spinal cord at 2, 3 and 4 months following ALS onset using transgenic FUS mouse model (FUS-TG). HPLC-MS was performed on a TSQ Endura (Thermo Scientific) instrument in multiple reaction monitoring (MRM) mode. The instrument was equipped with a EclipsePlusC8 column. The MRM transitions are shown in the scheme. Calibration curves were obtained using sphingosine, deuterated sphingosine, sphinganine and sphingosine-1-phosphate and proprietary mixtures of ceramides (Porcine Brain Ceramide) and sphingomielins (Porcine Brain Sphingomielin) with known compositions, both obtained from Avanti Polar Lipids, Inc.

Our results demonstrated sharp increase of such sphingoid bases as sphingosine and sphinganine, which are highly proapoptotic, in spinal cord cells at the terminal ALS stage. A deeper undestanding of biological pathways regulating sphingolipid metabolism in ALS is important for identifying new therapeutic targets.

FCS and FLIM methods combination for caspase-3 FRET sensor

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Keywords: FCS, FLIM, FRET sensor, photoconvertible FP, caspase.

Fluorescent Correlation Spectroscopy (FCS) allows determine diffusion coefficients of fluorescent molecules in the focal microscope volume. We can obtain information about interactions of labeled proteins or changes in the oligomeric state. Classical application of the proteins labeled with fluorescent proteins (FPs) is usually associated with the use of CMV promoter and therefore overexpression. However, FCS method needs low amount of fluorescent dye – near nanomolar concentrations. To control the amount of the fluorescent dye we used new photoconvertible FP SAASoti that was previously successfully monomerized in our group [1]. The advantage of this approach is that partial photoconversion of the FP makes FCS measurements when studying enzymatic reactions possible.

On the example of the apoptosis, we investigated caspase-3 activation during its early stages. To investigate the process *in vivo* we used HeLa cell line expressing the engineered FRET sensor – SAASoti-23-KFP – that is based on the TagRFP-23-KFP FRET sensor [2], which was effectively used in our recent studies. This FRET sensor has a cleavable (-DEVD-) sequence in the linker between two FPs. The apoptosis was induced by the addition of staurosporine. Caspase-3 activity was detected by registration the changes in the SAASoti fluorescence lifetime. We planned to confirm sensor cleavage by FCS measurements. SAASoti-23-KFP has a tetrameric structure due to KFP tetrameric state. After the sensor's cleavage proceeds SAASoti changes its oligomeric state and therefore the diffusion coefficient. Free monomeric SAASoti must be detectable by FCS measurements and we could quantify the ratio between the free SAASoti and its being a part of the sensor.

Surprisingly, after few hours of staurosporine was added, fluorescent lifetimes of the sensor increased, meaning its cleavage by caspase-3, whereas the diffusion coefficient of SAASoti decreased. It can be explained by an increase in the total cell viscosity during apoptosis. We can supposed that in the moment of detectible caspase-3 activity cells structure have already crucial changes.

This work was supported by Russian Science Foundation grant №19-14-00373.

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Cell membrane permeability and integrity monitoring using firefly luciferase bioluminescent system

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Keywords: firefly luciferase, cell membrane, eukariotic cells, membrane permeability

Firefly luciferase is a perfect label to use in different cellular processes monitoring. It is easy to detect and to deliver into cell with help of genetic engineering. The aim of this work was to study the effect of different agents on cell membrane permeability and integrity using firefly luciferase reporter. The gene of luciferase was inserted into the plasmid, intended for eukaryotic cells transfection. HEK-293 cells were transfected using lipofectamine with high efficiency. Procedures of cell lysis and luciferase activity measurements were optimised.

The effects of concentration and exposition time on cell membrane integrity were studied by measuring luciferase signal in cell supernatant. The system also allowed to measure ATP rate inside and outside cells, which is also an important cell physiology marker. It was found that unmodified luciferin is unable to get inside cells under physiological conditions, which makes it clear, that obtained signals come from reactions, catalyzed by luciferase released from damaged cells.

Kinetic curves of cell membrane destruction were obtained for different effectors. It was studied, whether they affect the enzyme, to exclude the effect of luciferase inactivation. The method was optimized to be used in compounds screening and membrane studies. It was proven with different types of membrane affecting compounds – saponins, antibiotics and substances, which cause oxidative stress. Digitonin was shown to cause fast destruction of cell membrane due to interaction with intramembrane cholesterol. Polymyxin, which is often used to treat infections, caused by gram-negative microorganisms, showed slower kinetics of membrane destruction. Both effectors had linear dependence on concentration in the studied interval. Agents of oxidative stress, which are reported to damage membranes, demonstrated no active effect nor in short, neither in long periods of time.

Magnetic nanoparticles in cancer therapy and diagnostics

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Keywords: iron oxide, MRI, drug delivery,

Magnetic nanoparticles (MNP) are in the field of great interest in two past decades. MNP can be used as an effective MRI contrast agents, drug delivery systems and effective formulations for magnetic hyperthermia. Such variety of application in each particular case can be reached only by specific chemical design of magnetic core structure and surface coating. Thus optimization of such parameters allows using these nanoparticles not only in separate application, but also in combined modalities.

For combined drug delivery and MRI imaging we have developed complex system based on iron oxide nanocrystals, coated with human serum albumin (HSA-MNP) with following crosslinking with formation of stable biocompatible shell. Physicochemical properties of HSA-MNP were investigated in details by HAADF-TEM, DLS, AFM, also magnetization and T2 relaxation properties were investigated. HSA serves as a natural transport protein for xenobiotics in blood and can effectively bind drug molecules to surface. Our experiments have shown that HSA-MNP were able to bind doxorubucin, cisplatin and bacteriochlorine *a* (PS) molecules, effectively deliver this drugs to tumor cells and tissue. Particularly for doxorubicin loaded nanoparticles we have shown effective imaging of 4T1 mouse breast cancer model accompanied with increase of median survival from 26 to 39 days.

PS loaded HSA-MNP has shown similar photoinduced cytotoxicity in comparison with free drug and were stable in water solution for few weeks. Moreover *in vivo* experiments with mice bearing tumors have shown that after *i.v.* injection of PS loaded HSA-MNP we were able to detect PS delivery to tumor by both MRI and *in vivo* fluorescence. Also it was shown that PS loaded MNP can be used as a predictor for proper time management of PDT in vivo.

This results allow to propose HSA coated MNP as a perspective tool for drug delivery of different antitumor drugs for cancer treatment.

The application of porous silver surfaces and nanoparticles in virus detection and identification *via* SERS

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Keywords: SERS, viruses, nanoparticles, identification, nanoplasmonic

The application of several silver SERS [1] substrates in direct label-free virus detection and identification was studied. One such SERS substrate was produced using electron-beam physical vapor deposition method (EB-PVD). By tuning the temperature during the deposition process it was possible to manufacture porous SERS-active surfaces with varying pore sizes. The possibility of using such silver substrates for virus detection was demonstrated by testing them against 4 viral species – 2 animal viruses, rabbit myxomatosis virus (MYXV) and canine distemper virus (CDV), and 2 plant viruses, tobacco mosaic virus (TMV) and potato virus X (PVX) [2]. The specific SERS spectra of all 4 viruses were successfully observed and collected. The spectral data was then processed and subjected to principal component analysis (PCA) and linear discriminant analysis (LDA) in order to evaluate the discrimination between groups.

A classification model was composed and validated.

Using the classification model 100% accurate discrimination between viruses was achieved. The parts of spectral data that contributed the most to discrimination model were analyzed and compared to manual peak assignment in order to confirm that achieved discrimination was based on actual differences in virus structure between tested species.

SERS-active silver nanoparticles [3] were also used for detection and discrimination between MYXV and CDV. Because most viruses are negatively charged under physiological conditions, normal negatively charged silver colloids are ill-suited for virus detection. It was demonstrated that application of positively charged nanoparticles or positively charged aggregation inducing reagents like spermin could remedy that problem. The use of direct label-free SERS measurements for virus detection was thus shown to be possible and worthy of further study.

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The effects of Paclitaxel, Cytochalasin-D, Progerin on cancer cell compartments studied by elastic modulus measurement via Scanning Ion-Conductance Microscopy

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8 ICAPPIC Limited, London, United Kingdom

Keywords: SICM, single-cell analysis, cell stiffness, cell compartments

Elastic modulus measurement of single cell via Scanning Ion-conductance Microscopy (SICM) is a novel method of studying cell mechanical properties. Due to the work principle of SICM [Korchev et al., 2009], which is allow to topography mapping with lateral and vertical nanoscale resolution. Also, it's possible to provide simultaneously elastic modulus mapping, due to applying low stress on cell surface [Clarke et al., 2016], whose nature is intrinsic colloidal pressure between nanopipette tip and cell membrane. Nanoscale diameter of nanopipette tip allows to obtain elastic modulus distribution on different parts of single cell.

We report cell elastic modulus measurement of drug-induced alterations in cancer cell compartments studied by SICM, specifically, we measured fibrosarcoma cells (HT1080) transfectioned with Progerin, which is integrate in protein structure of nucleus membrane. Progerin was modified with GFP fluorescence dye (GFP-Progerin). Also, we analysed human prostate cancer cell line PC3 subjected with Paclitaxel for microtubulin stabilization and Cytochalasin-D for actin depolymerization.

Experiments with GFP-Progerin were provided in heterogeneous population of HT1080 with control and GFP-Progerin transfectioned cells. Control elastic modulus measurement shows ~1.7kPa and ~0.7kPa, when GFP-Progerin treated cells increased value only on nucleus area (~2kPa). In control and treated PC3 cells we measured elastic modulus upon the nucleus area and cytoplasm area, which are show two different values in control cells (~1.3kPa and ~0.8kPa, respectively). Measured elastic modulus after Paclitaxel treatment shows significantly increased elastic modulus value on nucleus area and cytoplasm area (~4kPa and ~1.8kPa), whereas Cytochalasin-D treatment reduced cell elastic modulus only on cytoplasm area (~0.5kPa).

As we can see, SICM-base measurement of elastic modulus shows different effects Paclitaxel, Cytochalasin-D, Progerin on cancer cell compartments, including actin, microtubulin and nucleus membrane, respectively. Drug-induced disruptions of these cell compartments lead to elastic modulus alteration, depending on inhibition mechanism.

The research was supported by Russian Science Foundation grant (SICM supported by project No. 19-79- 30062 and biological experiments supported by project No. 17-15-01290)

Nanocomposites Based On Carbon Nanotubes And Amphiphilic Diblock Copolymers: Preparation And Prospects Of Application As Biosensor Coatings

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Keywords: nanocomposite, carbon nanomaterials, amphiphilic polymers, biosensor, dispersing.

Modification of electrodes by nanocomposite coatings containing carbon nanomaterials (single- and multi-walled carbon nanotubes, graphene, graphene oxide, etc.) is widely applied for construction of highly-sensitive electrochemical sensor setups. Level and quality of integration of the carbon nanomaterial in the nanocomposite coating determines quality, sensitivity, and ultimately market competitiveness of a bioanalytical device. However, such materials are in general hardly dispersible (especially in aqueous media) that brings considerable challenges and limits their application for modification of the electrodes.

This work aims at preparation and characterization of the nanocomposite coatings based on the carbon nanotubes and amphiphilic ionic diblock copolymers containing both hydrophobic and hydrophilic (ionic) blocks. A hydrophobic segment of the diblock copolymer adsorbs onto surface of carbon nanomaterial particles while a hydrophilic one provides their dispersing and high colloidal stability in aqueous media. The overall hydrophilic-hydrophobic balance as well as the total charge and charge density of the amphiphilic ionic diblock copolymer can be varied by changing lengths of the hydrophobic and hydrophilic blocks as well as aqueous medium conditions (for example, pH) that allows preparation of carbon-polymer hybrid materials with desired properties.

In this contribution, the examples of dispersing of multi-walled carbon nanotubes in aqueous solutions of both cationic (polybutadiene-block-poly(2-(dimethylamino)ethyl methacrylate)) and anionic (poly(n-butyl acrylate)-block-poly(acrylic acid)) amphiphilic diblock copolymers are reported and the prospects of application of the prepared nanocomposite carbon-polymer sensor coatings on planar graphite electrodes for direct quantitative electrochemical analysis of biomolecules (cytochrome c, DNA) are considered.

This work was financially supported by the Russian Science Foundation (the grant № 18-44-04011).

Green synthesis nanoparticles of metal oxide in water extract of parsley

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Keywords: metal oxide nanoparticles, biological nanoparticle synthesis, green synthesis, plant extract, bimetallic particles

It has long been known that the synthesis of metal nanoparticles (MNPs) from metal salts in plant extracts is relatively simple process that occurs at room temperature. A number of nanopowders of metal oxides (Fe_2O_3, Co_3O_4, $CoFe_2O_4$) was synthesized in aqueous extract of leaves Parsley curly *Petroselinum crispum*. According to the UV-spectra, Co_3O_4 suspension at 370 nm, $CoFe_2O_4$ (synthesized in acidic medium), had small arm at 242 nm and peak at 292 nm. At the same time, particles of alkaline nature had plateau with 229 nm and small peak at 366 nm. According to the DSR analysis, the polydispersity index was less than 0.7, which indicates good quality of the preparations, and the ζ-potential of more than -35 mV indicates their stability in the ash. Scanning electron microscopy showed that Co_3O_4 powders are small cubic particles with sharp edges (from 20 to 100 nm) stuck together in large aggregates (~1 μm); $CoFe_2O_4$, synthesized at pH=2 – large monodisperse particles with a characteristic cut, and in an alkaline medium (pH=9) – particles with smaller diameter (from 30 to 100 nm) and without cut.

Mechanisms for implementing the biological effects of MNPs on plant organisms are shown on *Triticum vulgare* wheat. Seeds were disinfected and germinated in climatic chamber under 12-hour illumination, temperature 22±1 °C and humidity 80±5% for 48 hours. Then 5 ml of nanopowder suspensions were added in concentrations from 10^{-1} to 10^{-5} M. On day 14, we calculated the viability (V) of the cells using the vital dye Evans blue. To do this, we cut the roots off the stems and placed in the dye for 15 minutes at room temperature, after which they were washed with distilled water for 10 minutes and divided into segments – apical and basal. Microdrugs were visualized in the light mode of microscope (Micromed-3, Russia) and the total number of cells was calculated according to the number of stained cells. Plants treated with 50 μM H_2O_2 served as positive controls.

Analysis of the V of the roots of the seedlings of *T. vulgaris* showed that more intensive differential processes occur in the apical part under the influence of MNPs than in the basal area. In general, after $CoFe_2O_4$ was added to the medium (synthesized at pH=2) and Co_3O_4, there was a slight decrease in VC cells by only 12 and 15% relative to the intact plants, respectively, which is 4-10% lower than the positive control level (H_2O_2). Moreover, significant increase in the VC relative to the control samples was recorded after exposure to minimal doses (10^{-4} and 10^{-5} M) of Co_3O_4 and $CoFe_2O_4$ (pH=2) - to 5 and 3%, respectively.

In turn, the maximum decrease in the index was recorded in response to the impact of $CoFe_2O_4$, synthesized in an alkaline medium, to more than 50%, with the number of dead cells in the basal and apical parts of the samples being 29 and 36% more relative to H_2O_2.

Study of magnetic field parameters influence on exosomal membrane fluidity in complexes with magnetic nanorods by fluorescent spectroscopy

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Keywords: exosomes, magnetic nanorods, fluorescent spectroscopy, FRET

Nowadays scientists are interested in study new perspective drug delivery systems. Among all of them membrane vesicles are very popular, particularly exosomes. Exosomes are produced by almost any cells in the organism and could be found in many biological fluids. Their possible application is extremely wide as they are non-toxic, biocompatible and have highly developed surface. The last property looks perspective for creation a drug delivery system with active targeting. Despite the advantages of exosomes, the issue of trigger drug release is still remains unsolved. As a possible solution we propose utilizing complexes of exosomes with magnetic nanorods. Exposed to the low frequency alternative magnetic field nanoparticles could oscillate and loose membrane of vesicles, promoting drug release.

The aim of the work was to study the influence of magnetic field parameters on exosomal membrane fluidity. As an effective instrument for such study we chose fluorescent spectroscopy. The approach is provided by inclusion of fluorescent dye into exosomal membrane. In this work we have used BODIPY labeled DPPC lipid derivative (B9PPC – BODIPY). The analytical signal is fluorescence polarization; its decrease evidence about membrane loosening. As an alternative fluorescent method we used Forster resonance energy transfer (FRET). For this two labels were incorporated into membrane vesicles: B9PPC and TMB-PC. Together they made a pair, where one label is an energy donor, and another one – energy acceptor. If these labels locate close to each other, the fluorescence starts to quench. Thus, increase of membrane fluidity could be noticed by decrease of fluorescent intensity.

Using FRET-analysis we have shown that indeed disordering of the exosomal membrane in complexes with nanorods occurs in five minutes exposure to magnetic field. The increase of magnetic induction led to a slight increase of membrane fluidity. However, there was no significant dependence between frequency of magnetic field and membrane loosening depth. The fluorescence polarization study appeared to be more informative. We have studied dependence between time exposure in magnetic field and loosening depth under different conditions (magnetic induction, field frequency). Every pair of magnetic parameters has its own time of maximal loosening. This time decreased with magnetic induction increase and field frequency decrease.

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Nanomaterials based on hydroxyapatite and carboxycellulose for drug delivery

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Drug delivery to a biological target is a fundamental part of the medicine development. For this purpose, various methods are used, among which the packaging of the main drug in biologically safe containers of different biodegradable composition takes an essential place. Such packaging is especially important for radiopharmaceuticals, including the ions of metal radionuclides or their complexes.

Hydroxyapatite (HAP) has a composition close to that of human bones, is able to integrate into the corresponding tissue and has a high sorption capacity. Particularly interesting is the enzymatic production of HAP using alkaline phosphatase. This method, studied in detail in this work, makes it possible to increase the sorption capacity of nanoparticles and allows obtaining material with predetermined particle sizes depending on the concentration of the enzyme used. The latter is extremely important, since the uncontrollability of sizes is the main obstacle to the use of nanoparticles in medicine. HAP samples carrying the copper and zinc ions, their complexes with cytotoxic derivatives of thiazine and aminopyrimidine, as well as the ^{69m}Zn and ^{64,67}Cu radionuclides were obtained. The cytotoxicity of HAP samples of different composition was investigated.

Another promising "packaging" material is carboxymethylcellulose with various quantities of metal ions introduced. Methods for obtaining such materials, their cytotoxicity with respect to normal and leukemic cells, are considered. Based on the characteristics of the materials obtained, the most convenient for further use were selected.

Superoxide dismutase nanoparticles for the treatment of inflammatory eye diseases

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Keywords: superoxide dismutase, nanoparticles, uveitis, antioxidant enzyme, inflammation

Currently, an active search for new drugs to treat inflammatory ocular diseases occurs. Oxidative stress plays an important role in the pathogenesis of inflammatory diseases, and injection of antioxidants may be effective. Antioxidant enzymes, such as superoxide dismutase (SOD) and catalase, have much more efficiency in comparison with small molecular antioxidants. However, administering native enzymes to the eye in the form of eye drops is ineffective due to their rapid clearance. Therefore, it is important to create a drug delivery system that will possess long time of circulation and low immunogenicity.

To achieve this goal, SOD nanoparticles covered with chitosan were synthesized. Polymeric shell was used to decrease immunogenicity; chitosan was used to increase time of circulation.

Briefly, SOD nanoparticles were synthesized by mixing of SOD solution with protamine and PLE-PEG solutions sequentially after that glutaraldehyde was added. Byproducts were removed by centrifugation through centrifugal filters.

The release experiments were conducted using a dialysis container (100 kDa). SOD had being released from nanoparticles more slowly than the native SOD. Thus, 90% SOD had released after 24 hours from the solution with the native enzyme, for the same time 30% SOD had released from the solution with nanoparticles. This demonstrates that SOD is connected with the polymers strongly and the particles may have a large circulation time as opposed to the native enzyme.

It was demonstrated that SOD nanoparticles and SOD nanoparticles coated with chitosan, first, are better retained on the surface of the mucous membrane of rabbits' eyes, compared with the solution of the native enzyme, second, they are able to penetrate into the internal structures of the eye, therefore it is possible to inactivate superoxide radicals inside eyes. Thus, superoxide dismutase nanoparticles covered with chitosan seem to be perspective therapeutic agent due to improved stability and ability to internalize into eyes.

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The development of magnetic liposomes-based nanoparticles iron oxide with different anisomeria

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Keywords: magnetic nanoparticles, magnetic liposomes, low-frequency alternating magnetic field

Biocompatible magnetic nanoparticles (MNPs) have a great potential in the field of pharmaceuticals and biomedical research. In particular, MNPs are used in the development of containers for drug delivery. Their application helps to solve the topical problems such as drug inactivation and degradation in the bloodstream.

Different types of nanocontainers are investigated to solve this issue. Liposomes are one of the promising and well-studied forms of nanocontainer. This type of containers delivers both hydrophilic and hydrophobic molecules of various sizes, but one of the significant drawback is the slow release or complete lack of the release of active molecules. To date, an approach to simplify and improve the release of the drug from liposomal containers, which are functionalized with MNPs under the action of low-frequency alternating magnetic field (LF AMF). The approach is based on the mechanical rotation of the MNPs in the LF AMF, leading to disordering of the lipid membrane of the nanocontainer.

The aim of the work was to obtain and study a model for the release of molecules from liposomes with immobilized MNPs of various shapes – "spheres" and "rods" - on their surface under the exposures to LF AMF.

Within the framework of this study, the following problems are considered: synthesis of MNPs by thermal decomposition for spherical particles and two-stage synthesis (hydrolysis and microwave radiation) for MNPs in the form of rods; preparation of magnetic liposomes by hydration of the lipid film; immobilization of MNPs, which are stabilized by nitrodophamine, to carboxyl groups, which are located on the surface of the liposomes, by carbodiimide method; study of membrane permeability under the LF AMF on the kinetics of suppression of the enzymatic reaction with alpha-chymotrypsin and the method of ATR-FTIR.

As a result of the work, MNPs of iron oxide of spherical shape and in the form of rods were synthesized. Magnetic liposomes with the size of 200±15 nm and low PDI were obtained. Under the action of LF AMF (110 Hz, 90 mT), the release of high molecular weight protein (the Bowman-Birk Inhibitor, 6-8 kDa) from magnetic liposomes with immobilized spherical MNPs were observed. In the case of MNPs in the form of rods, there is no statistically significant effect of AMF action, but there is a tendency to improve protein release in comparison to control experiments without field stimulation. According to ATR FTIR, MNPs that were immobilized on the surface of liposomes interact mainly with lipid phosphates and polyethylene glycol "coat". In this case, there was no disordering of the lipid membrane under the action of LF AMF observed. Nevertheless, the effect observed indicates the release of protein from the surface of the liposomes membrane. The possibility of release of protein due to the magneto-mechanical actuation of MNPs in the LF AMF was demonstrated.

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Aggregation of hybrid magnetite-gold nanoparticles modified with enzyme and inhibitor under low-frequency magnetic field

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Magnetic nanoparticles are one of the perspective carriers for creating new materials for biotechnology and biomedicine. Due to large surface area and large surface-to-volume ratio magnetic nanoparticles are of interest for biomacromolecules immobilization onto their surface. And the possibility to control behavior of such nanocomposite by external magnetic field opens opportunity for creating new remotely controlled materials.

In our work, we immobilized α-chymotrypsin (CT) and Bowman-Birk inhibitor (BBI) onto hybrid magnetite-gold nanoparticles (MGMNP), covered with PEG and lipoic acid. Under impact of 50 Hz magnetic field, a decrease of the enzymatic activity in CT-BBI couple, depending on magnetic field intensity (fig 1 A-C) was observed. No changes observed in the case of free CT immobilized onto MGMNPs (fig 1D). This effect seems to be related to aggregation of large nanoparticles under low-frequency magnetic field.

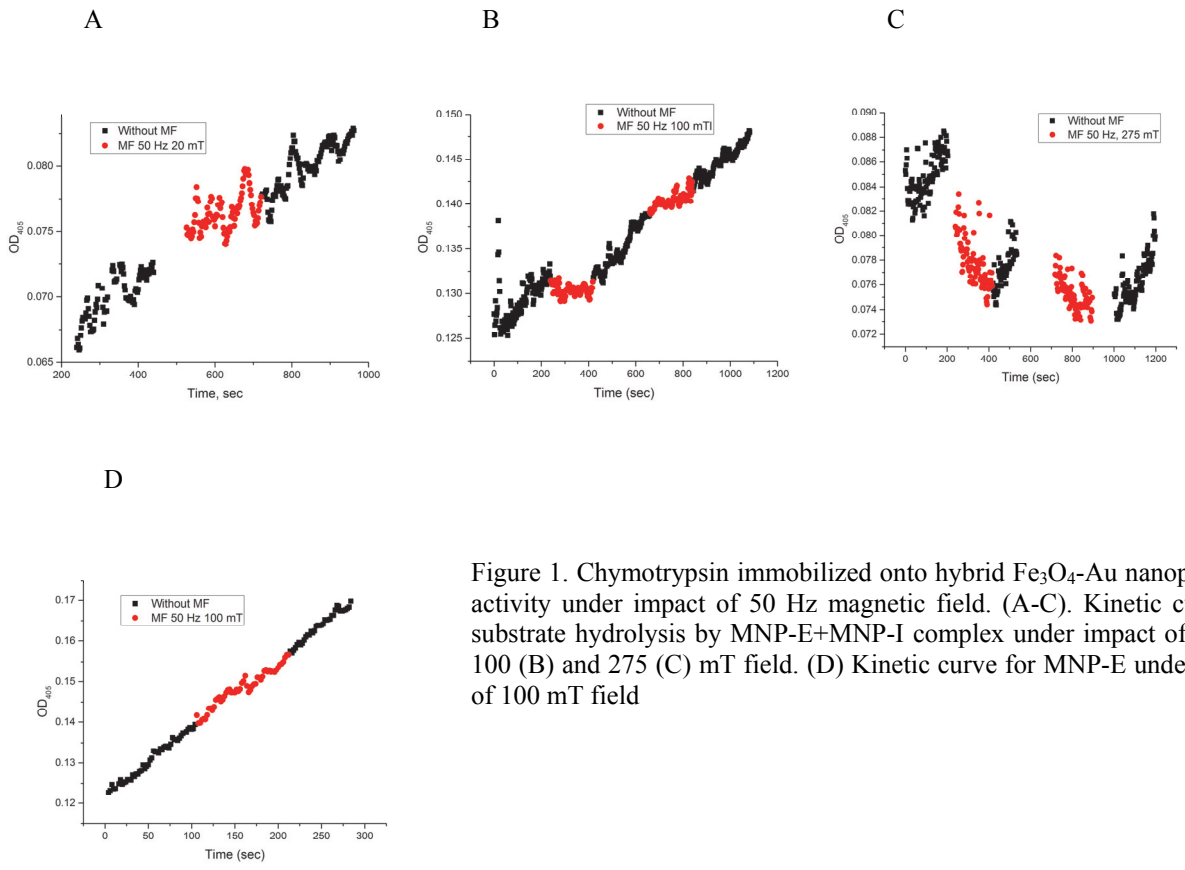

Figure 1. Chymotrypsin immobilized onto hybrid Fe_3O_4-Au nanoparticles; activity under impact of 50 Hz magnetic field. (A-C). Kinetic curves of substrate hydrolysis by MNP-E+MNP-I complex under impact of 20 (A), 100 (B) and 275 (C) mT field. (D) Kinetic curve for MNP-E under impact of 100 mT field

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