POSTERS

P1

DOMAIN-SWAPPING OF THE ANCESTOR OF HALOALKANE DEHALOGENASE AND *RENILLA* LUCIFERASE

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Domain swapping is a mechanism for two or more protein molecules to form a dimer or higher oligomer by exchanging an identical structural element. This phenomenon holds additional interest because it can serve as a mechanism for reversible oligomerization, and conceivably for pathological oligomerization, such as amyloidogenesis leading to neurodegenerative diseases. Several possible mechanisms of the domain swapping were described in the literature. The high energy barrier existing between closed monomer and the domain-swapped oligomer can be reduced by modification of pH, temperature, introduction of mutations, and presence of denaturants or binding of ligands [1].

Initially, we inferred the most probable sequence of ancestral enzyme AncHLD-RLuc of haloalkane dehalogenase (HLD) LinB and *Renilla* luciferase (RLuc). The gene encoding the predicted sequence was synthesized, expressed in *Escherichia coli*, and the resurrected enzyme was purified by metallo-affinity chromatography [2]. X-ray structural analysis revealed that AncHLD-RLuc can adopt two different conformations: monomeric conformation, very similar to that of LinB and RLuc monomers, and domain-swapped dimer conformation, which has never been detected before for any HLD member or RLuc. The quaternary structure of AncHLD-RLuc was therefore characterized in more details by size exclusion chromatography equipped with static light scattering, refractive index, ultraviolet and differential viscometer detectors. The changes in its quaternary structure were monitored during the long-term incubation at 40°C.

The analysis of the oligomeric states revealed that AncHLD-RLuc exists under tested conditions in the equilibrium between monomer, two types of dimer, and tetramer. The hydrodynamic radius and intrinsic viscosity of presented dimers uncovered different arrangement of their quaternary structure and thus verified the presence of previously identified domain-swapped dimer. The long-term incubation revealed that the monomer is highly stable during whole incubation at 40°C. On the contrary, the concentration of the domain-swapped dimer decreased after 4 hours of incubation and subsequently increased after 6 hours of incubation at 40 °C, while the classical dimer and tetramer slowly aggregated during whole thermal incubation experiment. The results demonstrated that the domain-swapped dimer is created from the less stable classical dimer. Furthermore, the stability of AncHLD-RLuc tetramer indicates the structural similarity with the classical dimer. The size exclusion chromatography with tetra detectors system is a powerful tool for the studying mechanisms of enzyme oligomerization.

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P2

STRUCTURAL DETERMINATION OF TOXIC AND NONTOXIC HYPF-N OLIGOMERS

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Oligomers formed by the N-terminal domain of *E. coli* HypF (HypF-N) are considered an important model for investigating the structure of misfolded protein oligomers involved in neurodegenerative disorders, particularly Alzheimer's disease. Indeed, such oligomers have morphological, structural and tinctorial features similar to those formed by proteins involved in diseases and impair cell viability in neuronal cells and neurons both *in vitro* and *in vivo*. Two types of stable HypF-N oligomers were previously formed in vitro, showing similar morphological and tinctorial features, but structurally disclosing different degrees of packing within their cores and different toxicities.

In this work we attempted to characterise the toxic type A and nontoxic type B oligomers of HypF-N in detail using solid-state NMR and site-directed fluorophore-labelling coupled to fluorescence resonance energy transfer (FRET) to report on intermolecular distances between 12 different positions within the two oligomer types. The aim of the work is to obtain information on the structure and dynamics of the two oligomeric forms at the level of individual residues and interactions and identify the specific structural elements and sites responsible for their difference of toxicity.

P3

UNFOLDING MECHANISM OF HUMAN FIBROBLAST GROWTH FACTOR 2 AND ITS COMPUTATIONALLY DESIGNED THERMOSTABLE VARIANTS

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Human fibroblast growth factor 2 (FGF2) serves as a regulator of proliferation, migration, and differentiation in a variety of human cell types. Studies revealed its potential use in the treatment of cancer, cardiovascular diseases and mood disorders [1-3]. Moreover, FGF2 is efficient in wound and epithelium healing and is used as an essential component of media for human stem cells cultivation [4-6]. Such versatile roles of the FGF2 protein make it a very promising target for biotechnological applications. However, its extensive use in these applications is hindered by its short activity half-life (10 hrs at 37 °C). Recently, two highly stable variants of FGF2 have been computationally designed, constructed and characterized in our laboratory. Both showed dramatically prolonged half-lives to several days, whilst preserving their biological activity [7].

Complex unfolding study of the wild type and the two constructed variants was performed to obtain deeper understanding of stabilizing effects of introduced mutations. The thermal unfolding mechanisms of the three FGF2 molecules were analysed by different biophysical techniques. The global fit of resulting data obtained by DSC, CD, fluorescence spectroscopy and nanoDSF revealed a two-step unfolding mechanism including one intermediate for the wild-type and both variants. Based on the estimates of the energy barriers it was concluded that the stability of both variants can be attributed to the increase in the Gibbs free energy of activation of the first unfolding step. These results perfectly correlate with the ones obtained from chemical denaturation experiments, where the kinetics of unfolding in urea was followed by using CD.

This study demonstrates how the combination of several biophysical techniques together with global mathematical analysis of the data helps to resolve multiple-step unfolding mechanism from the simple two-state one. Each

technique follows a unique structural property and provides a different view into the structural changes occurring during protein transitions. Moreover, this approach enables quantification of the individual unfolding steps in terms of energetics and thus permits to make important conclusions about the introduces changes of the stabilized variants.

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P4

ADENYLOSUCCINATE SYNTHETASE FROM BACTERIUM *HELICOBACTER PYLORI* STRAIN 26695: PURIFICATION AND CHARACTERIZATION

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Helicobacter pylori is a Gram-negative, microaerophilic bacterium, known for its ability to colonize human stomach and to participate in development of many diseases as gastric ulcers and stomach cancer [1]. Study of the *H. pylori*, due to the ever growing infection rate and increase of *H. pylori* antibiotic resistance, is centred on understanding pathogenesis and finding a way to attack and eradicate *H. pylori*.

Adenylosuccinate synthetase (AdSS) is one of the key enzymes in purine salvage pathway. It catalyses a reversible reaction utilizing IMP, GTP and aspartate in the presence of Mg^{2+} to form adenylosuccinate, GDP and inorganic phosphate. *H. pylori* AdSS represents potential drug target as this bacterium cannot synthesize purine rings through *de novo* pathway and has to rely on purine production through purine salvage pathway [2].

Adenylosuccinate synthetase gene *purA* was isolated from genomic DNA of *Helicobacter pylori* (strain 26695) and amplified using Phusion High-Fidelity PCR kit with the set of specific DNA primers for both 5' and 3' ends of the gene. Plasmid pET21b-HP26695*purA* was constructed by cloning *H. pylori purA* gene into pET21b expression vector, with ampicillin resistance and without purification tag. This plasmid was transformed into *E. coli* strain BL21-CodonPlus(DE3)RIL. Standard IPTG-induction conditions were used for AdSS expression in *E. coli*.

Purification of overexpressed AdSS from the bacterial protein extract was performed by cation exchange chromatography on SP-Sepharose FF column, followed by size-exclusion chromatography (SEC) on Sephacryl S-200 column. Final step, which gave single protein band on SDS-PAGE was fast protein liquid chromatography (FPLC), performed on anion exchange MonoQ 5/50 GL column.

Enzyme's molecular weight was estimated by SDS-PAGE and elution volume from SEC. It was concluded that in applied conditions enzyme exists in solution as a dimer. Kinetic studies involving all three substrates (IMP, GTP and aspartate) were conducted. Protein crystals appeared in several conditions of used commercial crystallization screens. Structure solving of adenylosuccinate synthetase from *H. pylori* is under way.

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P5

CRYSTALLIZATION OF REPRESSORS DEOR AND C-GNTR FROM *BACILLUS SUBTILIS*

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Bacterial transcriptional repressors are regulatory proteins acting as molecular switches of gene expression. The mechanism of the regulation is based on repressor binding to specific DNA operator sequences by its N-terminal DNA-binding domain (DBD) and blocking the transcription of genes. Their function is modulated by the binding of an effector molecule to its C-terminal effector-binding domain (EBD). Understanding the structure-function relationship and molecular mechanisms by which individual repressors execute their regulatory functions greatly benefits from knowledge of their 3D structures. In this project, we selected two repressors, which are involved in carbon catabolite repression, as targets for structural characterization.

The DeoR protein from *Bacillus subtilis* negatively regulates the expression of catabolic genes for the utilization of deoxyribonucleosides and deoxyribose. The structure of the EBD was determined in free form and in covalent complex with its effector [1]. Our aim is a determination of structure of full-length DeoR in complex with its DNA operator. The recombinant protein was expressed in host bacteria *E. coli* BL21(DE3). For the purification of the protein we used an immobilized nickel affinity chromatography, N-terminal His-tag was removed by TEV protease cleavage. The yield of the protein preparation was 2.9 mg from 1 L of bacterial culture. The complex of DeoR with its DNA operator (18bp) was prepared by mixing of the protein and DNA in ratio 1:1.1. The presence of the DNA in the crystals was proven by fluorescence microscopy. Crystallization screening followed by optimization yielded needle-shaped monocrystals diffracting to resolution 7Ĺ at a synchrotron radiation source. Optimization of crystallization conditions is now in progress.

The GntR protein from *B. subtilis* is a negative regulator which is responsible for gluconate metabolism in *B. subtilis*. Its structure has not been resolved yet. Our aim is to determine its EBD in complex with its effector gluconate. For recombinant expression and protein purification was performed similarly to DeoR described above. The yield was 0.9 mg of purified protein from 1 L of bacterial culture. Crystallization of GntR EBD in the presence of high molar excess of gluconate yielded hexagonal monocrystals with maximal dimension of 30 30 3 μm. Optimization of crystallization conditions is now in progress.

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P6

CHARACTERIZATION AND INITIAL CRYSTALLIZATION STUDIES OF THE HEAT SHOCK PROTEIN HSP104 AND ITS VARIANT WITH MODIFIED LENGTH OF DOMAIN M

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The process in which a newly synthesized polypeptide chain transforms itself into a perfectly folded protein depends both on the properties of the amino-acid sequence and on multiple contributing influences from the crowded cellular milieu. Uncovering the mechanism of protein folding and unfolding is one of the grand challenges of modern science. The three-dimensional arrangement of the polypeptide chain decides about the specific biological function of the protein in the cell. Only correctly folded proteins are fully functional, randomly arranged polypeptide chain doesn't have biological activity. The state of protein folding is controlled and regulated by the protein quality control system. The system is formed by chaperones involved in protein folding and the proteasomal degradation system. The proper functioning of the system is required because its dysfunction may lead to neurodegenerative diseases. The prion-related illnesses such as Creutzfeldt-Jakob disease, amyloid-related illnesses such as Alzheimer's disease as well as intracytoplasmic aggregation diseases such as Huntington's and Parkinson's disease those are neurodegenerative diseases whose pathogenesis is associated with protein aggregation of incorrectly folded proteins.

Many chaperones are heat shock proteins. Their expression is increased when cells are exposed to elevated temperatures or other stress conditions. The project focuses on the protein Hsp104 which belongs to the Hsp100 family and the AAA+ superfamily. Hsp104 is important in the cell due to its ability to solubilize and refold proteins trapped in aggregates formed during heat stress [1]. It achieves this in cooperation with the Hsp70 chaperone system. The active form of the protein is a ring-shaped hexamer, which is thought to drive protein disaggregation by directly translocating substrates through its central channel. However, there is still no general consensus regarding the domain organization within the hexameric molecular machine. Substantial efforts have been made to elucidate the location of domain M, but the results are contradictory [2, 3].

Results of characterization and initial crystallization studies of the heat shock protein Hsp104 and its variant with modified length of domain M will be presented.

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P7

RUTINOSIDASE – LABYRINTHINE JOURNEY FOR X-RAY STRUCTURE

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Obtaining well diffracting crystals and solving protein structure can be tedious work and successful process may include various crystallization techniques and tricks. Here we present one didactic story of crystallization -**L**-Rhamnosyl- -**D**-glucosidase (Rutionsidase) from *Aspergilus niger*. During the crystallization process, we performed screening using vapour diffusion method, optimization by counter diffusion technique, and final crystals soaking of heavy atoms in micro batch experiments, which allowed structure solution by SIRAS. However, to repeat the crystal growth, we had to deglycosylate the enzyme and perform new screening followed by Matrix Microseed Screening. Moreover, as final reproducible procedure, for growing the protein crystals, we used under oil micro batch experiments. With this optimised method, we were able to grow crystal that diffracted up to 1.27Å resolution and see structural details that shall be used in the future.

P8

STRUCTURE ANALYSIS OF HALOALKANE DEHALOGENASE DbeA CL VARIANT FROM *BRADYRHIZOBIUM ELKANII* USDA94

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A novel enzyme, DbeA, belonging to the family of haloalkane dehalogenases (EC 3.8.1.5) was isolated from *Bradyrhizobium elkanii* USDA94. This haloalkane dehalogenase is closely related to DbjA enzyme from *Bradyrhizobium japonicum* USDA110 (71% sequence identity), but has different biochemical properties. DbeA is generally less active and has a higher specificity towards brominated and iodinated compounds than DbjA. The DbeA protein was crystallised using the sitting-drop vapour-diffusion method and the crystal structure of a DbeA enzyme has been solved and deposited at Worldwide Protein Data Bank under PDB ID 4k2a. The DbeA wt structure revealed the presence of two halide-binding sites. The first chloride-binding site is located in the active site in between two halide-stabilizing residues. The second chloride-binding site is unique to DbeA and has not been previously reported in any other structure of this enzyme family. To elucidate the role of the second halide-binding site, a two-point variant DbeA Cl (I44L+Q102H) lacking this site was constructed and biochemically characterized [1]. Elimination of the second halide-binding site decreased the stability and catalytic activity, and dramatically altered the substrate specificity. The two-point substitution resulted in a shift of the substrate-specificity class, which is the first time this has been demonstrated for this enzyme family. Rational design of buried halide-binding sites represents a novel strategy for engineering of enzymes with modified catalytic properties.

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P9

EXPRESSION AND STRUCTURAL CHARACTERIZATION OF HALOALKANE DEHALOGENASES DhmeA AND DhcA

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Oligomerization plays an important role in the function of many proteins and is often related to the surrounding conditions of the protein environment [1]. From the perspective of structural biology, protein–protein interactions have mainly been analyzed in terms of the biophysical nature and evolution of protein interfaces [2]. Oligomerization can have a positive impact on protein functionality, leading to the formation of stable and functional multimeric protein, or negative impact, leading to diseases such as Amyloidosis, Alzheimer´s or Parkinson´s disease [3]. Here we investigated the model protein family of haloalkane dehalogenases (HLDs), which has been phylogenetically divided into three subfamilies HLD-I, HLD-II and HLD-III [4]. Contrary to the HLD-I and HLD-II subfamilies, from which most of so-far characterised HLD members originated, the HLD-III subfamily has not been structurally explored due to difficulties in enzyme production. Preliminary testing revealed that HLD-III members occur in high molecular weight clusters with an average Mw 1 MDa, exhibiting low activity when compared to other HLD members [5].

This project is focused on optimisation of protein production and structural characterization of selected enzymes belonging to HLD-III subfamily: DhmeA from halophilic archea *Haloferax mediterranei* ATCC 3350 and DhcA from halophilic marine bacterium *Hahella chejuensis* KCTC 2396. Both enzymes were expressed with and without fusion protein ubiquitin in *Escherichia coli* BL21(DE3) and cultivated in Luria broth, Terrific broth or EnPressoB medium under various temperatures. Solubility of expressed proteins was further optimized by using various buffers with different pH and additives during disintegration step. The quaternary structures of DhmeA and DhcA were analyzed by size-exclusion chromatography equipped with static light scattering, refractive index, ultraviolet and differential viscometer detector determining the protein size and dynamic light scattering estimating hydrodynamic radius of the proteins. Cryo-electron microscopy was used to assess DhmeA quaternary structure in its close to native state. Simultaneously, several crystallisation trials with DhmeA and DhcA were performed, however, no crystals suitable for X-ray diffraction experiments were obtained. One of the possible explanations could be the polydisperse character of the proteins. Future experiments will focus on increasing monodispersity of the protein sample.

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P10

STRUCTURAL AND BIOCHEMICAL CHARACTERIZATION OF TRANSCRIPTIONAL REGULATOR LUTR FROM *BACILLUS SUBTILIS*

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Metabolic transcriptional regulators are proteins controlling transcription of specific genes involved in bacterial metabolism. These proteins typically consist of two domains, C-terminal effector-binding domain (EBD) and N-terminal DNA-binding domain (DBD). When an effector (usually a small metabolite) binds to the EBD, protein changes conformation and either binds to DNA (in the case of activator) or leaves it (in the case of repressor). The presence or absence of the regulator on DNA operator then affects transcription of regulated genes.

LutR, formerly YvfI [1], belongs to the GntR family of bacterial transcriptional regulators. In undomesticated strain RO-NN-1 and probably many other strains of *Bacillus subtilis* LutR regulates transcription of genes required for L-lactate utilization [2, 3]. Interestingly, LutR from laboratory strains PY79 and 168 lacks the first 21 amino acids (Met1 – Asp21, designated here as LutR 1_21del) and plays a role as a global regulator [3, 4]. In these domesticated strains LutR 1_21del positively or negatively regulates many genes associated with transition from exponential growth to stationary phase [4]. The missing 21 amino-acid residues long fragment includes a part of the predicted winged helix-turn-helix DNA-binding motif and deletion in this region apparently alters DNA sequence specificity.

To fully understand the impact of this short deletion on the mechanism of DNA recognition we initiated structural studies of both LutR variants. Our aim is to characterize structure of protein-DNA complex for both LutR and LutR 1_21del by X-ray crystallography and NMR. We plan to work with single domains (DBD) as well as with full-length proteins. We optimised protocols for heterologous expression of proteins in soluble form in *Escherichia coli* and subsequent purification protocol using immobilized chelate affinity chromatography. Sufficient amounts of proteins for further crystallization and biochemical experiments were prepared. Yields of unlabelled and ^{15}N- or SeMet-labeled proteins ranged between 12.8 – 85.8 mg/l of bacterial culture and 3.5 – 18.5 mg/l of bacterial culture, respectively. We are using electrophoretic mobility shift assay, size exclusion chromatography and NMR spectroscopy for characterization of protein-DNA complex properties. We initiated crystallization experiments and obtained preliminary crystals for LutR, LutR 1_21del and complex of LutR DBD with DNA operator. Further crystallization trials and optimization of crystallization conditions are in progress.

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P11

STRUCTURAL CHARACTERIZATION OF THE NEW HLD I SUBFAMILY MEMBER WITH THE UNIQUE PROPERTIES

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Haloalkane dehalogenases (HLDs; EC 3.8.1.5) are enzymes with broad potential application in biocatalysis, bioremediation, biosensing and cell imaging as a consequence of their property to catalyse hydrolytic cleavage of halogen-carbon bond in a wide range of aliphatic halogenated hydrocarbons and their derivatives via SN_2 nucleophilic substitution followed by addition of water, releasing halide ion, proton, and the corresponding alcohol as the reaction products [1; 2].

A new HLD DpcA from *Psychrobacter cryohalolentis* K5 is allocated among of all biochemically characterized HLDs by it's unique property -the highest observed activity at 25 °C (with retained almost 27 % of its maximal activity at 5 °C) and the narrowest substrate specificity profiles .That property makes DpcA valuable source of using it at conditions which are not suitable for another HLDs.

To analyse DpcA structure, the enzyme was crystallized [4] ($P2_1$ space group) and the data diffraction were collected on the beamline 14.2 at the BESSY II electron-storage ring (Helmholtz-Zentrum Berlin (HZB), Germany), equipped with a Rayonics MX-225 CCD detector at the wavelengths of 0.978 Å. The data were refined to the resolution 1.05 Å and phases were solved by *MOLREP* from the CCP4 software suite using the coordinates of *Xanthobacter autotrophicus* (PDB code: 1B6G; 40% sequence identities for 121 residues and 53% sequence similarity).

DpcA crystall structure confirmed DLS and 1H NMR spectrum results showing the monomeric globular form. The structure composed of two domains. The active site is burried between them and is typical for HLD-I subfamily: nucleophile D123, catalytic base H280, catalytic acid D250 and halide-stabilizing W124 and W164.

As the enzyme stabilization without diluting and decreasing it's activity is crucial for the the industrial catalysts [5], the first HLD cross-linked enzyme crystals (CLECs) of were obtained and DpcA CLECs specific activity was examined towards 1-bromohexane, towards which the native enzyme has the highest specific activity.

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DETERMINATION OF PROTEIN-LIGAND INTERACTIONS USING DIFFERENTIAL SCANNING FLUORIMETRY

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A wide range of methods are currently available for determining the dissociation constant between a protein and interacting small molecules. However, most of these require access to specialist equipment, and often require a degree of expertise to effectively establish reliable experiments and analyze data. Differential scanning fluorimetry (DSF) is being increasingly used as a robust method for initial screening of proteins for interacting small molecules, either for identifying physiological partners or for hit discovery. This technique has the advantage that it requires only a PCR machine suitable for quantitative PCR, and so suitable instrumentation is available in most institutions; an excellent range of protocols are already available; and there are strong precedents in the literature for multiple uses of the method. Past work has proposed several means of calculating dissociation constants from DSF data, but these are mathematically demanding. Here, we demonstrate a method for estimating dissociation constants from a moderate amount of DSF experimental data. These data can typically be collected and analyzed within a single day. We demonstrate how different models can be used to fit data collected from simple binding events, and where cooperative binding or independent binding sites are present. Finally, we present an example of data analysis in a case where standard models do not apply. These methods are illustrated with data collected on commercially available control proteins, and two proteins from our research program. Overall, our method provides a straightforward way for researchers to rapidly gain further insight into protein-ligand interactions using DSF.

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CONFORMATIONAL AND ASSEMBLY DYNAMICS OF PROTEINS STUDIED BY TIME RESOLVED SMALL ANGLE X-RAY SCATTERING COMBINED WITH THZ SPECTROSCOPY

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Many proteins rely on conformational plasticity to perform intricate structural and biochemical tasks. This plasticity can be the motion of domains in single proteins or the reorientation of the subunits within a complex. In both cases the overall change in shape of the particle can be monitored by small angle X-ray scattering (SAXS). High brilliance synchrotron X-ray radiation allows for short exposure times and hence time-resolved detection of scattering data. Time-domain THz sources produce ultrashort electromagnetic pulses that can excite concerted molecular motions in energy ranges associated with domain or subunit conformational dynamics. In conjunction with time resolved shape information from SAXS, we aim to study the polymerization dynamics and dynamic plasticity of proteins. Microtubules are a fundamental component of the cytoskeleton with well-defined subunit plasticity and polymerization dynamics and are therefore an interesting model system for such investigations. We designed a flow cell

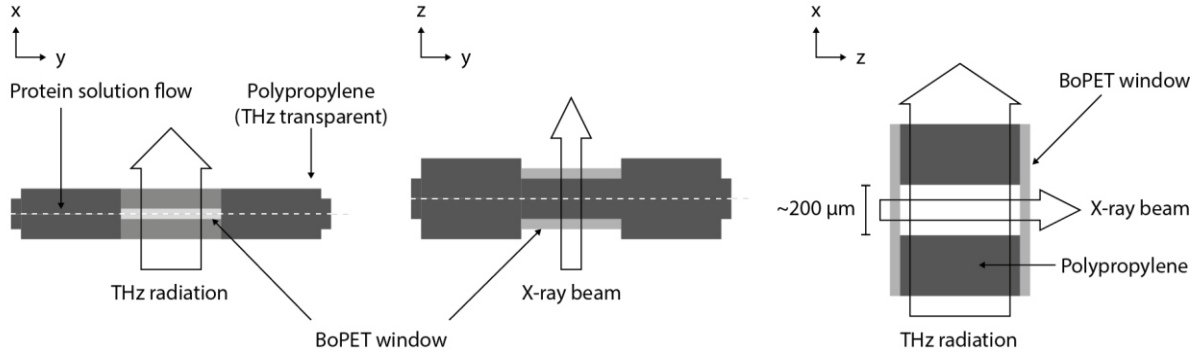

Figure 1. Design sketch of the THz- and X-ray-compatible flow cell. The chamber prototype is made of 3D-printed, THz-transparent polypropylene with windows made of biaxially-oriented polyethylene terephthalate (BoPET) for X-ray transmission.

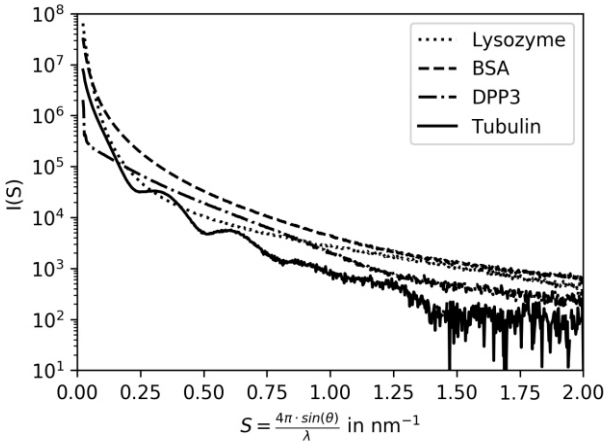

Figure 2. SAXS profiles recorded at the P12 BioSAXS beamline at the EMBL outstation in Hamburg, Germany. Tests of the flow cell with lysozyme, bovine serum albumin and human dipeptidyl peptidase 3 (DPP3) are shown alongside the scattering of Taxol-stabilized microtubules.

that is compatible with both, X-ray and THz radiation to enable a study using simultaneously both frequency regimes. Due to the strong absorption of THz radiation by water, the THz optical path has to be minimized.

We present the proof of principle of our sample chamber design with a static X-ray scattering experiment of Taxol-stabilized microtubules and other proteins. Future designs will include constant flow for time resolved measurement, temperature control and simultaneous irradiation of the sample chamber with THz radiation perpendicular to the X-ray path. The implications of excitable motions in protein mono- and polymers are manifold: Enzymatic activity, substrate binding and assembly-disassembly events associated with conformational changes could be observed under non-equilibrium conditions, providing deeper insight into their respective mechanisms.

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