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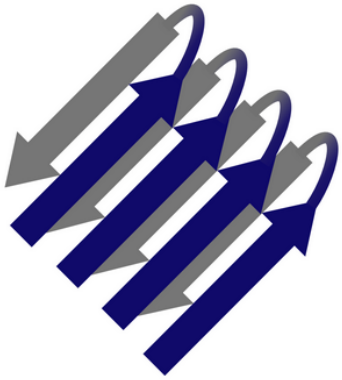

ADAM9

Amyloid Diseases and Amyloid Mechanisms

Riga, 14th–16th November 2022

Abstract Book

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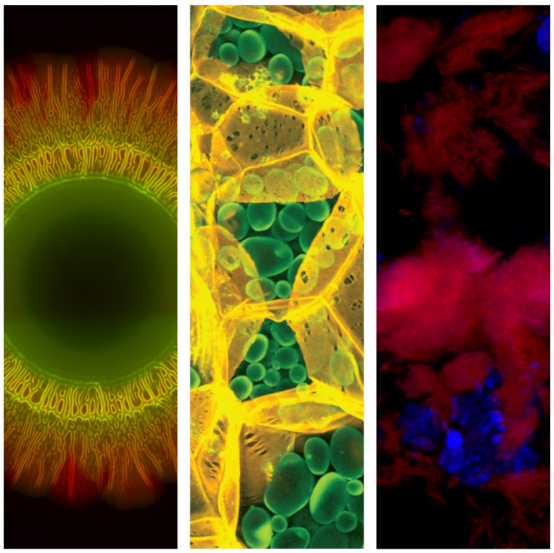

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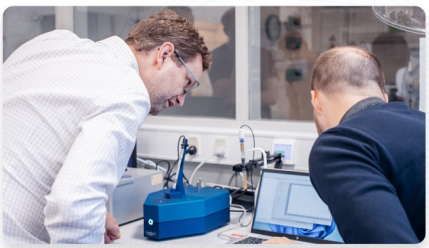

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Dear conference participants,

We welcome you to the **9th Scandinavian and Baltic Conference, Amyloid Diseases and Amyloid Mechanisms (ADAM 9)**. The conference was initially planned for 2020, but had to be postponed by two years due to the COVID-19 pandemic, and we are very happy that we can finally meet in person. As you know, amyloid is associated with approximately 30 human diseases including the incurable Alzheimer's disease, which has also been referred to as a silent, neglected pandemic. This reaffirms the importance of our research and the meeting.

This year **the conference will be devoted to commemorating Professor Sir Christopher Dobson**, one of the pioneers of protein folding and misfolding research in the area of amyloid diseases. Chris worked in the University of Oxford from 1980 to 2000 as a Fellow and then Professor of the Lady Margaret Hall and Department of Chemistry. He moved to the University of Cambridge in 2001 as the John Humphrey Plummer Professor of Chemical and Structural Biology. In 2007, Chris became the Master of St John's College, Cambridge, a post which he held until his death in September 2019.

Chris transformed the amyloid field using his enormous insights within protein structure, stability and dynamics. His contributions established a systematic and conceptually consistent basis for amyloid research and his Center for Misfolding Diseases at Cambridge University, now continued by Tuomas Knowles and Michele Vendruscolo, became the preeminent place to go to understand amyloid – particularly from the mechanistic and molecular perspective. Chris authored and co-authored more than 800 articles and inspired not lesser number of scientists, who worked in his laboratory and with whom he maintained close contacts. He was a tireless and enthusiastic champion of amyloid research and his warm and witty personality forged networks between scientists all over the world. The conference participants are encouraged to share photos or memories of him at the conference.

Message from his wife Mary Dobson: "What special news about the Conference in Riga on 'Amyloid diseases and amyloid mechanisms 9' dedicated to Chris' memory. What an incredible gesture and a really fitting tribute to Chris".

We are proud to host the ADAM 9 at the University of Latvia, the leading research university in Latvia. The conference is organised in collaboration with the Latvian Institute of Organic Synthesis, the leading drug discovery and development centre in the Baltics. We hope that you will enjoy the conference and have inspiring discussions at the posters and during the social events leading to new collaborations and excellent science. We wish you a pleasant stay in Riga!

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Evidence for human transmission of amyloid-β pathology

Jaunmuktane, Zane[1]

[1] University College London, UK

Introduction: The hallmark neurodegenerative disease known to be transmissible between humans is prion disease. Experimental seeding of amyloid-β (Aβ) and other misfolded proteins has been demonstrated in animal models, but if person to person transmission of misfolded proteins or peptides other than prion has occurred through medical treatments was not known up until 2015.

Objectives: Here we show that Aβ proteopathic seeds, similar to misfolded prion protein, are transmissible through medical procedures and treatments.

Material and methods: We performed an observational autopsy study of eight iatrogenic Creutzfeldt-Jakob disease (iCJD) patients and 116 patients with prion diseases of different aetiologies. Next, the pathology archive was searched for young adults with histologically confirmed cerebral amyloid angiopathy (CAA) and a control group of 50 consecutive age-matched patients. Extent and type of Aβ and tau pathology were assessed histologically and previous medical interventions were identified from the clinical notes. In all patients the *APP*, *PSEN1*, *PSEN2* were screened and *APOE* polymorphism determined.

Results: In the autopsy cohort, we found Aβ pathology in half of the patients. Aβ deposition in the grey matter was typical of that seen in Alzheimer's disease and Aβ in the blood vessels was characteristic of CAA. Four patients with CAA and history of neurosurgery several decades earlier were identified on review of the archival CAA material. Three had undergone diagnostic brain biopsy for investigation of intracerebral haemorrhage and one had died of complications from intracerebral haemorrhage. None had mutations in genes associated with early Aβ pathology.

Conclusion: Over the last seven years, strong evidence has been gathered indicating that Aβ is transmissible in humans through medical procedures with contaminated surgical instruments and treatments, involving human cadaver-derived tissues, such as growth hormone administration and dura mater grafting.

Intravenous treatment of Alzheimer disease mouse models with a recombinant BRICHOS domain improves pathology in relation to its efficiency against neurotoxic Aβ42 oligomers *in vitro*

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Background: Attempts to treat Alzheimer's disease (AD) with immunotherapy against the amyloid-β peptide (Aβ) or with enzyme inhibitors to reduce Aβ production have not yet resulted in an effective treatment, suggesting that alternative strategies may be useful. Recombinant human (rh) Bri2 BRICHOS can inhibit Aβ42 fibril formation, and it prevents neurotoxicity of Aβ42, both in hippocampal slice preparations and in a *Drosophila melanogaster* fly model.[1] Rh Bri2 BRICHOS can also pass the blood brain barrier in mice and rescue already established Aβ42 induced deterioration of hippocampal neural network activity *in vitro*,[2] suggesting that it could be a means to treat AD.

Questions addressed: We aimed to explore the possibility to target the toxicity associated with Aβ42 aggregation *in vivo* by using a blood brain barrier permeable rh Bri2 BRICHOS chaperone domain, mutated to selectively reduce Aβ42 oligomer generation and alleviate Aβ42-induced neurotoxicity in hippocampal slice preparations.

Methods: We treated Aβ precursor protein (App) knock-in mouse models with repeated intravenous injections of saline (controls) or rh Bri2 BRICHOS R221E,[3] and analysed cognition, Bri2 BRICHOS amounts in the CNS, Aβ plaque load and neuroinflammation after the end of treatment period.

Results and discussion: Rh Bri2 BRICHOS R221E treatment improved recognition and working memory, somewhat reduced Aβ plaque deposition, but substantially reduced astrocyte accumulation in the vicinity of Aβ plaques (Figure 1). The degrees of treatment effects observed correlate (i) with the amounts of Bri2 BRICHOS detected in brain sections after the end of the treatment period and (ii) with rh Bri2 BRICHOS R221E effects on formation of Aβ toxic oligomers and hippocampal neural network activity *in vitro*. This is the first study showing the effects of intravenous treatment with a designed BRICHOS domain in mouse models and the results motivate further work to enable treatment of AD. The apparent correlation between effects on Aβ42 toxic oligomer generation *in vitro* and effects on AD like pathology *in vivo* support the notion that specific inhibition of Aβ42 neurotoxicity is more relevant than reducing overall amyloid fibril formation.

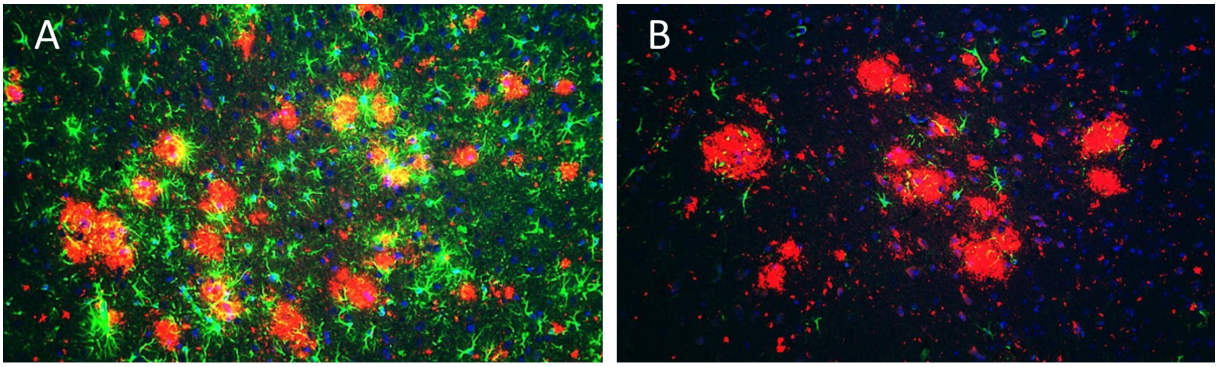

Figure 1. Immunohistochemical staining of Aβ plaques (red) and activated astrocytes (green) in cortex after intravenous treatment of App knock-in mice with (A) saline or (B) rh Bri2 BRICHOS R221E for ten weeks. (Manchanda et al, unpublished).

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Designing a Novel *Drosophila* Model of Alzheimer's Disease to Study Aβ Proteotoxicity in the Digestive Tract

Elovsson, Greta[1], Klingstedt, Therése[1], Brown, Mikaela[1], Nilsson, K. Peter R[1], Brorsson, Ann-Christin[1]

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Background: Amyloid-β (Aβ) proteotoxicity is associated with Alzheimer's disease (AD) and caused by protein aggregation resulting in neuronal damage in the brain. To combat this disease, there is a need to screen for anti-Aβ proteotoxic agents to discover and develop methods of treatment. *Drosophila melanogaster* is often used as a model of AD, where toxic Aβ peptides are expressed in the fly brain.[1]

Questions addressed: Since drug molecules often are administrated orally to the fly there is a risk that the compounds will not reach the brain, due their inability to pass the brain barrier. In this study, we have designed a novel *Drosophila* model of AD that expresses the Aβ peptides in the digestive tract. In addition, a built-in apoptotic sensor was exploited and provides a fluorescence signal from the green fluorescent protein (GFP) as a response to caspase activity, thus indicating apoptosis.[2] This gut-based *Drosophila* model is especially useful to screen for anti-Aβ proteotoxic compounds, since it assures that the orally administered drug reaches the target site where the toxic Aβ species reside.

Methods: Proteotoxic effects by expression of Aβ peptides in the fly gut were examined by a longevity assay, GFP fluorescence to identify apoptotic cells and by specific staining of Aβ aggregates using an antibody and the amyloid binding luminescent conjugated oligothiophene (LCO) h-FTAA.[3]

Results and discussion: The fly genotypes that we utilized in our study expressed either two copies of the Aβ1-42 peptide (Aβ1-42x2), one copy of a tandem Aβ1-42 dimeric construct (TandemAβ), or one copy of the Arctic mutation of the Aβ1-42 peptide in the fly gut. It was found that the amount of Aβ-aggregates correlated well with a higher mortality rate, where expression of the Arctic mutant of Aβ1-42 or TandemAβ generated the most toxic outcome, as shown in Figure 1. Additionally, all three Aβ-expressing fly genotypes showed a trend of a higher number of apoptotic cells compared to the control flies. Taken together, this gut based Aβ expressing fly model can be used to study the mechanism of Aβ proteotoxicity and to screen for novel therapeutic candidates to combat AD.

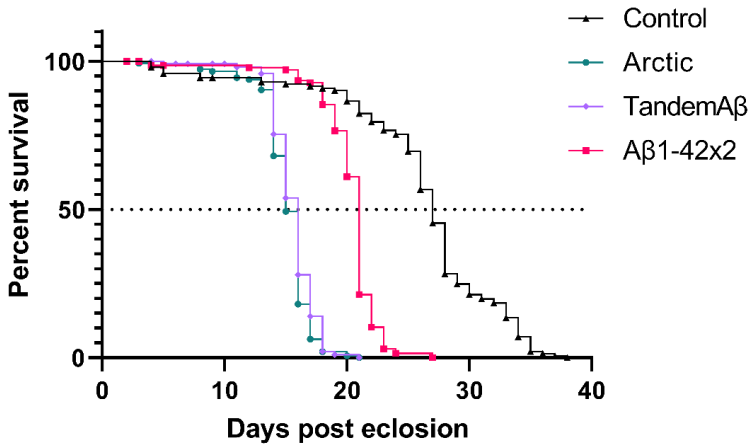

Figure 1. Longevity analyses showing toxic effects on Aβ expressing flies. Toxic effects were assessed by longevity assay for Arctic- (green, circle), TandemAβ- (purple, diamond), Aβ42x2- (pink, square), and control flies (black, triangle). Median survival times (50% survival) of Arctic-, TandemAβ-, Aβ1-42x2-, and control flies were 15, 16, 21, and 27 days, respectively.

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Use and abuse of functional amyloid: how to control and direct protein self-assembly

Daniel Otzen

Interdisciplinary Nanoscience Center (iNANO), Aarhus University, DK

In contrast to the pathological amyloid associated with neurodegenerative diseases, functional amyloid are a showcase of efficiency and usefulness found in many different organisms. They are particularly widespread in bacteria (FuBA) where work in particular on the *E. coli* curli and *Pseudomonas* Fap operons have uncovered simple and robust principles for controlled amyloid biogenesis. Fibrils, largely composed of one protein (*E. coli* CsgA or *Pseudomonas* FapC), extend from the surface of the outer membrane, assembled by transport through a dedicated export channel with associated anchoring proteins, periplasmic aggregation inhibitors and surface nucleators and increasing the mechanical strength of the bacterial biofilm. FuBA are optimized for aggregation through imperfect repeats, each of which can form one layer in a β-helix and lead to a fast -track fibrillation involving nucleation and elongation. Each of these steps involves essentially the same level of folding at the monomer level. Removal of the repeats increases the tendency to fragment and destabilize the fibrils. The amyloid structure is recapitulated and enhanced when the protein is presented with a structured surface such as graphene which is covered in a very comprehensive and highly regular fashion by the amyloid. FUBA's robustness notwithstanding, they are sensitive to inhibition, not only by generic amyloid blockers such as the polyphenol EGCG but also by designed peptides which divert the amyloidogenic proteins towards more amorphous and weakly organized aggregates. This inhibition reduces biofilm formation and opens up for more efficient intervention against bacterial growth in combination with more conventional antibiotics.

Functional amyloids seen by ^{1}H and ^{19}F detected solid-state NMR spectroscopy at fast magic angle spinning regime

Alons Lends

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Background: The amyloid fibrils are one of the main players to understand severe neurogenerative diseases at a molecular level. The solid-state NMR (ssNMR) spectroscopy has proved to be a powerful technique to resolve the atomic resolution structures of amyloid fibrils. The efficient high-quality sample preparation is a crucial step towards the structure determination by ssNMR.

Questions addressed: In this study we will apply protein cell-free (CF) synthesis method to prepare of selectively labeled self-assembled amyloids fibrils, for ssNMR spectroscopy at fast MAS regime, as well as for other techniques (electron microscopy (EM), X-ray diffraction (XRD), prion infectivity, ThT binding). We evaluated the CF synthesis using prion-forming domain HET-s(218-289) and truncated form of TAR DNA binding protein (TDP-43), TDP-11 (constituting the 99 amino acids from amyloid forming core).

Results and discussion: Using CF protein synthesis we manage to prepare functional amyloids in less than 20h with a high yield (1mg/mL of reaction mixture). In EM, the sample exhibited long, needle shaped type fibrils and had a high prion infectivity. In multidimensional ssNMR spectra the HETs(218-289) sample exhibited narrow ^{1}H peaks, similar to the same protein prepared using bacterial based expression system. Using tailored ^{1}H, ^{13}C and ^{15}N isotope labeling schemes we manage to assign chemical shifts and obtain distance restraints for the structure calculations. We integrated 5,5-difluoro-Leu in HETs (218-289) as a substituent for Leu in amino-acid mixture and apply the same CFPS conditions as show in **Fig 1A**. By using such approach we can use relatively small amounts of fluorinated amino acid (~5 mg for our CF syntheis method compare to 100 mg for cell-based methods The CF yield of of fluorinated was similar as before (1 mg/mL) with a high purity.

In conclusions, we have demonstrated here the potential of CF synthesis for a rapid preparation of high quality self-assembled amyloid fibrils for various fast MAS NMR studies and as well for other biophysical-methods. The CFPS method was validated using HETs(218-289) as the standards. The selectively labelled HETs(218-289) revealed a high quality and less overlapping spectra. We also successfully with the yield incorporated 5,5-difluoro-Leu for 19F detection of uniquely labelled Cd groups. Further on we are going to apply 2D and 3D methods for the extracting unambiguous 1H-1H and 19F-19F distance restraints and hydrophobic interactions as valuable assets for the structure determination and dynamic measurements of amyloid fibrils.

Nomenclature: Much more than names

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Kinetics of protein aggregation

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The role of environment in amyloid aggregation

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Background: The ability to form amyloid structures may be a generic property of polypeptides, and there are two major factors which define the probability of amyloid fibril formation – amino acid sequence of the protein/peptide and the environmental conditions. In the case of folded proteins, at least partial unfolding is necessary to trigger the amyloid formation pathway, so increased temperature, extreme pH conditions, addition of denaturants or any other changes in the environment leading to destabilization of protein structure are used in amyloid aggregation studies. Even in the case of disordered proteins, neutralization of charges or contact with hydrophobic surfaces may be necessary to induce amyloid formation. In addition to the specific conditions required for amyloid formation, changes in the environment may alter the mechanism of aggregation and lead to distinct amyloid fibril conformations. Finally, environment conditions affect the kinetics of aggregation and may alter the effect of anti-amyloid compounds.

Questions addressed: How environment conditions affect polymorphism of amyloid fibrils?

Methods: Thioflavin T (ThT) fluorescence assay was used to follow aggregation kinetics and for the initial suspicion of different fibril conformations. Fourier transform infrared (FTIR) spectroscopy was employed to discriminate different amyloid fibril conformations. Atomic force microscopy helped to get insight into fibril morphology.

Results and discussion: Studies of insulin aggregation at different conditions using FTIR spectroscopy allowed to identify at least four different conformations of insulin amyloid fibrils.[1,2] Further observations showed that deeper studies of ThT binding and fluorescence may be sufficient to discriminate between these conformations.[2] It opened the possibility to test much bigger number of samples and expand studies towards other proteins. Studies of prion protein showed that in the bigger pool of identically treated samples there are at least two populations of samples, which contain different amyloid fibril conformations.[3] Moreover, such environment-independent polymorphism seems to be present at the range of different temperatures.[4] Similar study of alpha synuclein showed similar picture at some conditions, however there were some conditions where only a single amyloid fibril conformation was detected.[5]

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Classification of Aβ amyloid fibril structures in mouse models of Alzheimer's disease

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The formation of fibrillar aggregate deposits from misfolded proteins known as amyloids is a pathological hallmark of neurodegenerative diseases. Alzheimer's disease (AD) pathology is characterized by the accumulation of Aβ peptides in senile plaques and tau proteins in neurofibrillary tangles**.** Apart from senile plaques and tau tangles, cerebral amyloid angiopathy (CAA) is also observed in several AD patients. CAA is formed by Aβ amyloid deposits within blood vessels. Albeit the disease-causing protein has the same primary sequence, the tertiary and quaternary fibrillar structures can be polymorphic. Previous studies showed that this structural polymorphism may correlate with different clinical phenotypes of AD.[1] Therefore, investigation of amyloid structural polymorphism is of great importance to understanding the disease mechanism.

In this present study, we wanted to explore how Aβ fibril morphology differs in Aβ plaque and CAA of different AD mouse models expressing familial AD mutations in the *APP* gene. To decipher the structural polymorphism, we utilized conformation -sensitive dye LCOs (Luminescence Conjugated Oligothiophenes), Aβ-specific antibodies (4G8 (Aβ epitope 18-22) and 12F4 (Aβ epitope 36-42), and different fluorescence microscopy techniques. The LCO fluorescence intensity ratiometric plot from the region of interest (ROI) showed different mouse genotypes have different fibril structures depending on transgenic genotypes. Co-staining of LCOs and Aβ-specific antibodies showed that the APP23 mouse genotype expressing the double Swedish mutations (NL) has a distinct plaque morphology compared to the APP knock-in mouse APPNL-F, expressing Iberian mutation (F) along with Swedish double mutation.

Individual APP23 plaque has two distinct fibril polymorph regions of core & corona. The plaque core consists of compact Aβ-40 fibrils, meanwhile, the corona consists of diffusely packed Aβ-40 fibrils. Very little Aβ-42 appears to be present in APP23 senile plaque. On the other hand, APPNL-F mouse plaque has a tiny core consisting of compact Aβ-42 fibrils. Both APP23 and APPNL-F mice have CAA composed of Aβ-42 fibrils bound to the blood vessel wall. These structural insights may help to identify disease-relevant fibril polymorph structures for high-resolution studies by CryoEM to guide the design of molecules for therapeutics.[2]

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S100A9 interacts with alpha-synuclein and alters the structure of amyloid fibrils

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Background: The formation of amyloid fibril plaques in the brain creates inflammation and neuron death. This process is observed in neurodegenerative disorders, such as Alzheimer's and Parkinson's diseases.[1] Alpha-synuclein (α-syn) is the main protein found in neuronal inclusions of patients who have suffered from Parkinson's disease.[2,3] S100A9 is a calcium-binding, pro-inflammation protein,[4] which is also found in such amyloid plaques.[5] To understand the influence of S100A9 on the aggregation of α-syn, we analyzed their co-aggregation kinetics and the resulting amyloid fibril structure. In addition, we analyzed the interaction sites between monomeric α-syn and S100A9. Here we show that α-syn interacts with S100A9 at specific regions, which are also essential in the first step of aggregation. We also demonstrate that 4-fluorophenylalanine label in α-syn is a sensitive probe to study its interactions and aggregation using ^{19}F NMR spectroscopy.

Questions addressed: Currently, there is limited data regarding cross-interaction of S100A9 and α-syn and how it influences the aggregation process. To understand the possible interaction between S100A9 and α-syn and influence on aggregation, we combine various methods to obtain low and high resolution details of the binding and aggregation.

Methods: In this work, we analyzed the interaction of S100A9 with α-syn using 1D ^{19}F and 2D ^{15}N–^{1}H HSQC NMR spectroscopy and molecular dynamics simulations. The aggregation kinetics, fibril structure, and toxicity of α-syn were analyzed by ThT fluorescence assay, atomic force microscopy (AFM), Fourier transformed infrared (FTIR) spectroscopy, solution and solid-state NMR spectroscopy, cell viability and membrane permeability tests.

Results and discussion: The results of this work showed that even small concentrations of S100A9 had a major influence on both the formation of α-syn fibrils, as well as their secondary structure and morphology. The ionic strength was important for S100A9 interaction with α-syn. S100A9 and high ionic strength could change the pathway of α-syn fibril formation, favoring a particular structure from the ensemble of structural populations. Additionally, we analysed the details of interaction between S100A9 and α-syn. Based on NMR data, we show that the N-terminal part of α-syn interacts with S100A9. MD simulations support this observation and allow selection of the possible complex structures. We also show that it is possible to study the interaction and aggregation of 4F-Phe labeled α-syn, which allows evaluating conformational changes and the stability of α-syn N-terminal and NAC parts separately. 2D ^{15}N–^{1}H HSQC NMR experiments support this finding and allow to obtain more details of amino acid residues involved into complex formation of S100A9 and α-syn. The NMR experiments provide details about α-syn during aggregation: 1) calcium stabilizes α-syn; 2) S100A9 accelerates aggregation of α-syn 3) the presence of calcium and S100A9 leads to a different α-syn aggregation pathway. Finally, we conclude that calcium and S100A9 influences the pathway of α-syn fibril formation and the final structure.

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The thermodynamics of amyloid fibril formation

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Background: While the kinetics of amyloid fibril formation has received widespread attention in the last two decades, much less work has been done to quantify the thermodynamic stability of amyloid fibrils. It is generally thought that amyloid fibrils are very stable under most conditions, and that therefore their formation is essentially a kinetic problem, i.e. the key question is not whether they form at all, but rather how fast (or slow). Nevertheless, the thermodynamic stability of amyloid fibrils could define to some extent their persistence in a biological context and therefore the study and quantification of this property is of relevance.[1]

Questions addressed: We will address the question as to how the thermodynamic stabilities of amyloid fibrils can be quantified. Furthermore, we will discuss the relative thermodynamic stabilities of different phases of aggregating proteins, including bulk[2] and nanoscale[3] liquid condensate phases.

Methods: The experimental methods employed are fluorescence spectroscopy,[4] capillary flow experiments (Capflex,[2]) and mass photometry.[3]

Results and discussion: We demonstrate that chemical depolymerisation, combined with intrinsic fluorescence spectroscopy is a convenient tool to accurately determine the thermodynamic stability of amyloid fibrils. Data from such experiments can be used to discriminate between different theoretical models of protein polymerisation appropriate for amyloid fibril formation.[4] Furthermore, we show that the conversion of liquid condensate droplets of alpha-synuclein into amyloid fibrils can be monitored by measuring the dilute phase concentration in (pseudo-)equilibrium with the droplet phase at different time points.[2] The dilute phase monomer concentration undergoes a significant drop when the reversible, liquid-like droplets are converted into amyloid fibrils.

Finally, we demonstrate that great care needs to be taken when phase diagrams are to be defined of proteins that can undergo both LLPS and convert into amyloid fibrils, as such systems are constantly evolving under most conditions. Furthermore, we use mass photometry to establish that nanoscale protein assemblies are present even under conditions where no microscopically visible droplets are forming, and that these nanoscale assemblies can evolve into amyloid fibrils concomitant to their slow growth.[3] The latter results have also been obtained with alpha-synuclein.

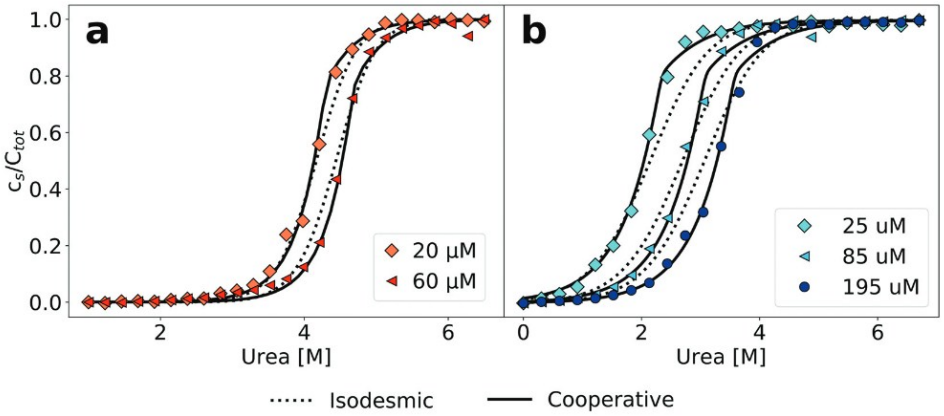

Figure 1. Cooperative polymerisation describes the concentration dependence of chemical depolymerization profiles of PI3K-SH3 (a) and glucagon (b) amyloid fibrils. The black lines show the best global fits of the two different linear polymerization models.[4]

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Amyloid as a generic structural form of proteins – new insights from food proteins

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Background: The field of amyloid research has developed from a strong focus on medical conditions to include also functional amyloid, nanotechnology and material science.[1] The wide variety of proteins that can be transformed into amyloid-like structures opens for many new applications but also raises critical questions regarding the differences and similarities in structure, formation mechanisms and biological activity for "artificial fibrils" compared to disease-associated amyloid.

Questions addressed: We have investigated the formation and the structures of amyloid fibrils from a range of food protein resources, including whey, egg, legumes, oat, rapeseed and potato.2–5 We also used kinetic assays to test if these fibrils could cross-seed a diseases related protein.

Methods: Fibril formation was studied by biophysical methods and imaging techniques. Fibril-forming segments in hydrolysed samples were identified by mass spectrometry.

Results and discussion: Amyloid-like structures were formed by all protein sources at low pH and elevated temperature and our data suggest that protein hydrolysis is a key step in the assembly process. The degree of hydrolysis may also define the morphology of the formed amyloid fibrils. The implication of these results for the use of amyloid-structures in materials and food will be discussed. Investigations of potential cross-seeding between food protein fibrils and amyloid b showed that none of the investigated food proteins accelerated amyloid b aggregation. This supports that amyloid-like structures are safe to use in materials design.

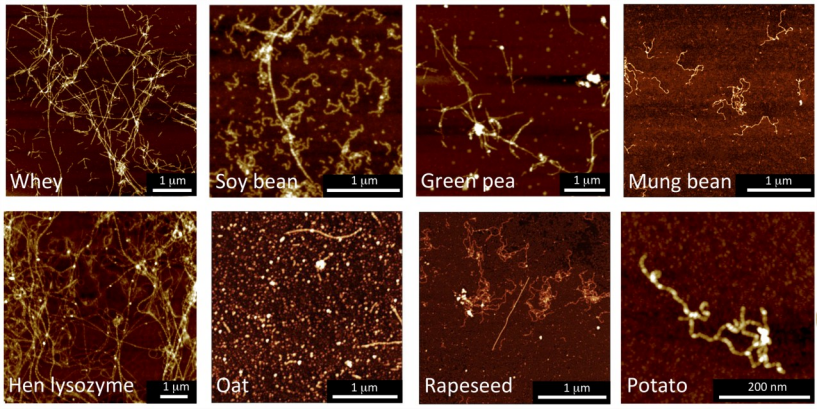

Figure 1. AFM images of amyloid-like nanofibrils from various protein resources.

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Tau protein phase transitions

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Background: The intrinsically disordered tau protein is known to aggregate into amyloid filaments involved in many neurodegenerative diseases, including Alzheimer's disease (AD) and Pick's disease. Amyloid aggregates are solid-like protein assemblies that are highly ordered and stable. Different aggregate structures are involved in distinct pathologies and seems to be able to propagate in a prion-like manner. Yet, the basic mechanisms or factors that drive tau aggregation and structural differentiation remains unknown. In the last few years, tau has been shown to undergo liquid-liquid phase separation (LLPS), either on its own or in complex with a polyanionic cofactor, such as RNA and heparin.

Questions addressed: Our research focuses on characterizing the transitions from tau soluble state to LLPS state to amyloid state. In particular, we investigate the physico-chemical properties that enable LLPS to evolve to amyloids.

Methods: We combine biochemical assays with biophysical methods, with a particular focus on electron paramagnetic resonance spectroscopy (EPR), to characterize tau aggregation pathways.

Results and discussion: In this work, we highlight that tau LLPS can rely on different types of interactions, hydrophobic and electrostatic. Depending on the dominant forces, the protein properties in the dense phase are drastically different. In particular, we show that electrostatically-driven LLPS have little impact on tau dynamics and structure,[1] and seems to be independent of aggregation. Conversely, tau LLPS based on hydrophobic interactions triggers drastic modifications in the physico-chemical properties of the protein, resulting in the promotion of amyloid aggregation.[2] This study provides an example showcasing that protein LLPS is a rich and complex modulator of biomolecule activities.

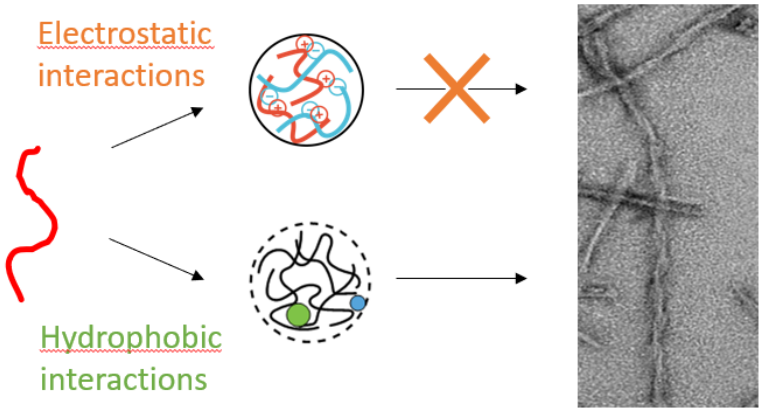

Figure 1. Different interactions can drive tau LLPS, leading to different pathways toward amyloid aggregates

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Aggregation Condition–Structure Relationship of Mouse Prion Protein Fibrils

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Background: A peculiar aspect of amyloid formation is the ability of one type of protein/peptide to form multiple, structurally distinct fibrils.[1] Lack of insight into this, seemingly generic, property of protein fibrillization may be one of the reasons why there is still no complete understanding of amyloid aggregation and, in turn, very few effective treatments for their respective diseases. In this work, we investigate mouse prion protein folding domain (MoPrP(89-230)).[2] We prepare fibrils in three different denaturing conditions and study them using FITR, AFM and MAS NMR. Based on the NMR chemical shift assignments we compare locations of secondary structure elements. Finally, we analyse our obtained data in conjunction with the three known structures of human prion protein fibrils to determine the structural implications of aggregation conditions.

Questions addressed: Which residues form the rigid core of MoPrP(89-230) fibrils? What are the overall differences between MoPrP(89-230) fibrils, prepared in different denaturation conditions?

Methods: Uniformly ^{13}C and ^{15}N labeled MoPrP(89-230) protein was expressed in *E. coli* and purified as described elsewhere.[3] The protein was fibrilized in 2 M GdnHCl with 50 mM sodium phosphate, pH 6.0, 4 M GdnHCl with 50 mM sodium phosphate, pH 6.0 and a mixture of 3 M urea and 1 M GdnHCl with 1× PBS, pH 7.4, each was seeded for two rounds. NMR, FITR and AFM data were acquired as described in the following publication of this study.[3]

Results and discussion: Combined data from NMR, FITR and AFM allowed for a comprehensive comparative analysis of the prepared fibrils. In all the samples, rigid core is formed by residues 165-223. The differences for residues preceding His177 and succeeding Tyr218 are negligible, indicating that the structure of N- and C-terminal regions of the fibrillar core is not affected by the aggregation condition. Both samples produced with GdnHCl as sole denaturant are more similar to each other and hence show small chemical shift deviations, except for the residues between Lys194 and Glu196. In contrast, in comparisons with the chemical shifts of the sample with urea, fibrils show larger differences, particularly in the regions Asp178–Asn181 and Lys204–Met213. Analysis of methyl group region of the NMR C(HH)C spectra propose that the denaturant ionic strength plays a major role in determining the structure of fibrils obtained in a particular condition by stabilizing fibril core interior-facing glutamic acid residues (Figure 1).

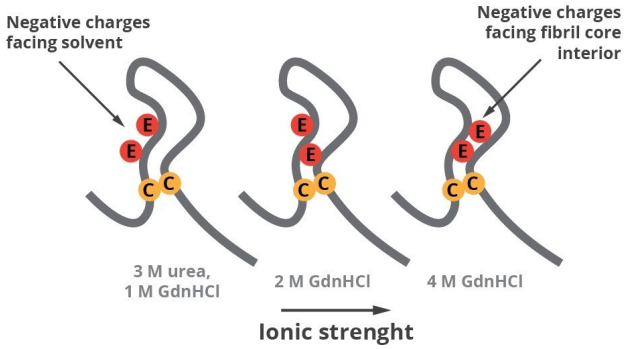

Figure 1. Dependence of glutamic acid location in the prion protein fibrils on the ionic strength of denaturation agent.

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Metals and membranes in amyloid formation and propagation

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Role of pro-inflammatory S100A9 protein in amyloid-neuroinflammatory cascade in neurodegenerative diseases.

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Background: The amyloid cascade and neuroinflammation are central to the onset of Alzheimer's and many other neurodegenerative diseases, however the mechanistic insights into how inflammatory events are linked to the amyloid formation remain unclear. S100A9 may serve as a critical link in the amyloid-neuroinflammatory cascade in these diseases, as this protein possesses both amyloidogenic properties and acts as an alarmin, triggering inflammatory responses.[1]

Questions addressed: We studied the interaction and co-aggregation mechanisms between S100A9 and Aβ peptides as main processes leading to amyloid plaques formation, neural cytotoxicity and tissue damage in Alzheimer's disease. The regulation of this process by small molecules is also addressed in the light of their therapeutic potential.

Methods: The kinetic analysis of amyloid aggregation by thioflavin-T fluorescence and Rayleigh scattering assays, microfluidic analyses, charge detection mass spectroscopy, AFM microscopy and molecular dynamic simulation were used in synergy in collaborative interdisciplinary research.

Results and discussion: We have found that S1009 protein is intrinsically amyloidogenic and able to form amyloids both *in vitro* and *in vivo* – i.e. in solution mimicking physiological conditions, in cell models and in the brain tissues during neurodegenerative diseases, including Alzheimer's, Parkinson's and traumatic brain injury. By using charge detection mass spectrometry in combination with AFM microscopy, kinetic analysis and microfluidic binding assay we have demonstrated that S100A9 co-assembles with $A\beta_{42}$ fibrils, forming a new type of hetero-amyloid complexes.[2] In these complexes the autocatalytic surfaces of $A\beta_{42}$ fibrils template S100A9 amyloids, where each component represents a homo-molecular domain in the hetero-molecular $A\beta_{42}$-S100A9 co-assembly. These change the dynamics of $A\beta_{42}$ amyloid aggregation and distribution of sizes of resulting co-assembled $A\beta_{42}$-S100A9 complexes. The formation of larger $A\beta_{42}$-S100A9 complexes may sequestrate smaller and more toxic species from the environment, which is consistent with our previous finding that co-aggregation of S100A9 with either $A\beta_{42}$ or A β_{40} mitigate the overall amyloid cytotoxicity. These findings contribute to understanding of amyloid co-aggregation processes both from a fundamental perspective and in revealing disease relevant processes.

Small molecules, regulating S100A9 amyloid aggregation and functions, including cell penetrating NCAM1 peptide constructs, oleuropein aglycone, Nb_{10} and $TiNb_9$ polyoxometalates,[3] cyclin and DOPA derivatives, are viewed in the light of their prospective therapeutic applications and also providing insight into specific sequences in S100A9 structure, which can drive or block its amyloid formation.

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Unravelling the Role of S100A9 in the Development of Neurodegenerative Disease.

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Background: S100A9 is a pro-inflammatory calcium binding protein released by neutrophils in response to neuro-inflammatory events. These events can be triggered by a range of conditions including traumatic brain injury and bacterial infection but are also observed in a range of neurodegenerative conditions including Alzheimer's Disease and Parkinson's Disease. S100A9 has been shown to be intrinsically amyloidogenic, readily forming fibrils *in vitro* and *in vivo*.[1] Interestingly the formation of S100A9 has been shown to facilitate the deposition of other amyloidogenic proteins including amyloid-beta peptide[2–4] and alpha-synuclein (aSN).[5–7] To understand the factors that initiate and promote S100A9 aggregation and determine how it mediates its effects on other amyloidogenic proteins we are investigating the structural transitions associated with S100A9 fibrilization and how the intermediates on the folding pathway interact with and modify the structure of other amyloid deposits.

Questions addressed: We have sought to investigate how changes in buffer/salt concentration influence the aggregation of S100A9 and, in turn, how this influences the interaction of other amyloidogenic proteins.

Methods: We have used an array of biophysical techniques to assess both the kinetics of S100A9 fibrilization and the structures present on the aggregation pathway. Furthermore, we have been able to study how these species interact with and influence the aggregate rate and structures formed of other amyloidogenic proteins. The influence of salt/buffer conditions on the structure of the monomeric protein has been assessed through a combination liquid-state NMR and synchrotron radiation CD (SRCD), whilst solid-state magic-angle spinning (MAS) NMR and transmission EM studies have proved invaluable insights into the amyloidogenic structures formed.

Results and discussion: The ionic environment to which S100A9 has a significant impact on the rate of S100A9 aggregation, with the presence of Ca^{2+} suppressing the rate of aggregation. Solution NMR and SRCD studies suggest that despite the presence of transient alpha-helical structures, in the absence of Ca^{2+} significant conformational exchange is still present. Binding of Ca^{2+} appears to suppress this conformational exchange and limits S100A9's ability to aggregate. Solid-state NMR and EM studies of the S100A9 structures formed over a range of Ca^{2+} concentrations indicate that despite the conformational exchange present in the soluble form of S100A9, upon fibrilization S100A9 adopts a single well defined conformational state existing as a single polymorph with approximately 85% of the protein present in a rigid amyloidogenic core, with the remaining residues possessing greater mobility.

Recent cryo-EM studies have indicated that strain/polymorph of aSN present may be a key discriminator as to the nature of the synucleinopathy presented by the patient.[8] Earlier studies have already demonstrated that S100A9 can enhance the rate of aggregation of alpha-synuclein.[5–7] To assess how elevated levels of S100A9 and S100A9 fibrils may influence the conformation state aSN adopts, proton-detected MAS-NMR studies were conducted in conjunction with TEM studies. These revealed that the presence of S100A9 not only accelerates the fibrilization of aSN but influences the type and nature of the aSN deposits formed, potentially impacting on the development of the disease.

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HSP10 as a Chaperone for Neurodegenerative Amyloid Fibrils

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Neurodegenerative diseases (NDs) are associated with accumulated misfolded proteins (MPs). MPs oligomerize and form multiple forms of amyloid fibril polymorphic structures that dictate fibril propagation and cellular dysfunction. Protein misfolding processes that impair protein homeostasis are implicated in onset and progression of NDs. A wide variety of molecular chaperones safeguard the cell from MP accumulation.[1] Paradoxically, chaperones have also been shown to promote fibril propagation of yeast prions.[2] A rather overlooked molecular chaperone is HSP10, known as a co-chaperone for HSP60. Due to the ubiquitous presence in human tissues and protein overabundance even compared to HSP60 we studied how HSP10 alone influence fibril formation in vitro of Alzheimer's disease associated MP Aβ1-42. At sub-stoichiometric concentrations, eucaryotic HSP10s (human and Drosophila) significantly influenced the fibril formation process and the fibril structure of Aβ1 -42, more so than the procaryotic HSP10 (GroES). Intriguingly at higher, but still substoichiometric, concentrations HSP10 delayed fibril formation by inhibited primary nucleation and fibril elongation, whilst at exceptionally low concentrations of HSP10 (<10 nM) fibril formation was accelerated (Figure 1). Similar effects were observed for prion disease associated MP HuPrP90-231. HSP10 is a homoheptameric co-chaperonin. We hypothesize that HSP10 by means of its seven mobile loops provides the chaperone with high avidity binding to amyloid fibril ends. The preserved sequence of the edge of the mobile loop GGIM(V)L (28-32 human numbering) normally dock to the chaperonin HSP60 apical domains. Interestingly this segment shows obvious sequence similarity to amyloidogenic core segments of Aβ1-42, GGVVI (37-41) and HuPrP90-231 GGYML (126-130) likely allowing efficient competitive binding fibrillar conformations of these MPs. Our results propose that HSP10 can function as an important molecular chaperone in human proteostasis in NDs and balancing HSP10 concentrations is of high importance.[3]

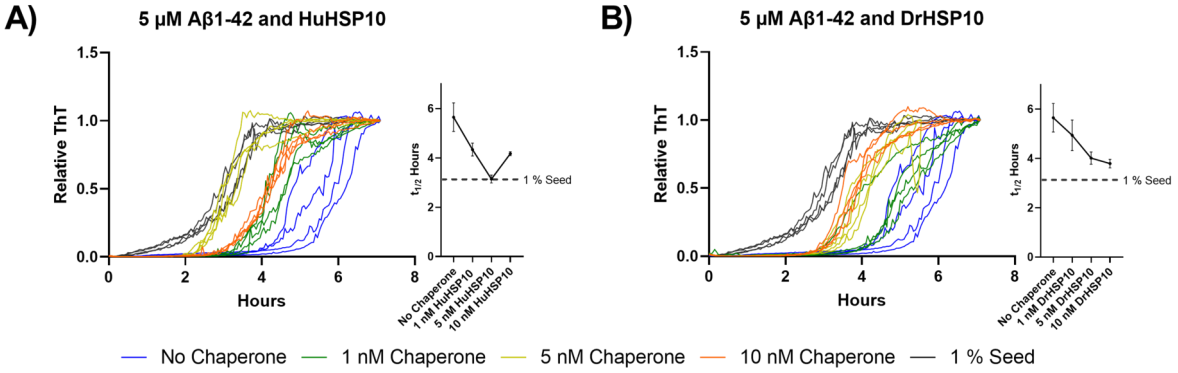

Figure 1. Normalized ThT traces of Aβ1–42 fibrillation (5 μM) when chaperones are present at low concentrations as well as Aβ1–42 fibrillated in the presence of 1% preformed fibril seeds. Traces are colored according to the legend in the figure. Corresponding halftimes of fibril conversion, $t_{1/2}$ (hours), for each concentration are shown to the right in each panel. **(A)** Aβ1–42 and HuHSP10 and **(B)** Aβ1–42 and DrHSP10. In this concentration interval, both HuHSP10 and DrHSP10 accelerate the fibrillation of Aβ1–42 compared with the spontaneous fibrillation of Aβ1–42 alone. The assay was conducted in quiescent conditions at 37°C in non-treated 96-well plates.[3]

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Interaction of protein aggregates with biological membranes: a key event in neurodegenerative diseases. From basic science to clinical trials

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Alzheimer's and Parkinson's diseases are neurodegenerative conditions affecting *ca.* 50 million people worldwide, and discovering effective treatments is therefore a medical emergency. These currently incurable conditions are characteristically associated with the aberrant deposition of proteinaceous aggregates in the brain, and formation of metastable intermediates known as protein misfolded oligomers thought to play a seminal role in their aetiology. The proteins involved are the amyloid b peptide (Ab) and a-synuclein in Alzheimer's and Parkinson's diseases, respectively.

I will describe a key event in such diseases, that is the interaction of protein oligomers with the membranes of neurons and how such interaction initiates a cascade of events culminating in neuronal dysfunction. I will also describe how steroid polyamines (or aminosterols) can target efficiently this interaction with a protective outcome. Indeed, steroid polyamines isolated from the entrails of sharks, namely squalamine and trodusquemine, have the ability to modulate the conversion of these two soluble proteins in nuclei to initiate the process of amyloid fibril formation and act also on secondary nucleation. They also have the ability to displace toxic oligomers from the cell membrane of cultured cells and lipid bilayers of reconstituted liposomes. These effects are mirrored in *C. elegans* animal models expressing Ab or a-synuclein and on the basis of this evidence clinical trials have started with promising results. Hence, based on an array of results at the physicochemical, molecular, cellular, animal model and clinical levels I will show how these natural products offer promising opportunities for chronic treatments for these progressive conditions.

Amyloidogenesis and cross seeding potential of SARS-COV-2 Spike protein

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Background: SARS-CoV-2 infection and long COVID is associated with several symptoms strikingly similar to blood coagulation and fibrinolytic disturbances[1] as well as neurologic and cardiac problems associated with amyloid disease. This led us to perform *in vitro* investigation of SARS-CoV-2 spike protein (S-protein) amyloidogenicity.[2] Spike derived peptides have been suggested to accelerate neurodegenerative diseases.[3] Aβ levels in CSF from patients suffering neurological complications after COVID-19 infection are similar to what is detected in Alzheimer´s disease.[4]

Questions addressed:
Is SARS-CoV-2 spike protein amyloidogenic? Can Spike derived amyloid effect blood coagulation?
Does Spike derived amyloid accelerate amyloid formation of proteins associated with neurodegenerative diseases?

Methods: Seven amyloidogenic amino acid sequences in the SARS-COV-2 S-protein were identified using the WALTZ algorithm. Corresponding peptides were synthesized and subjected to amyloid formation *in vitro*. The fibrillation kinetics was monitored by ThT and the endpoint samples were analyzed using transmission electron microscopy (TEM) and Congo red birefringence (CR). Recombinant expressed full-length S-protein was subjected to *in vitro* cleavage by neutrophil elastase (NE), a protease abundant at site of SARS-CoV-2 infection or mRNA vaccine injection.[5] Mass spectrometry was performed at endpoint of elastase cleavage to determine which peptides were abundant after intermediate and complete cleavage of S-protein. An *in vitro* fibrin(ogen)-fibrinolysis assay in absence and presence of Spike amyloids was performed and monitored by sample turbidity. Recombinant Aβ, Tau and PrP were subjected to seeding by Spike amyloid *in vitro*.

Results and discussion: Three 20-amino acid long synthetic spike peptides fulfilled our three amyloid fibril criteria: nucleation dependent polymerization kinetics by ThT, Congo red birefringence, and ultrastructural fibrillar morphology by TEM. All seven peptides fulfilled at least one of the three criteria. Full-length folded S-protein by itself did not form amyloid fibrils. However, amyloid-like fibrils with evident branching were formed during 24 h of S-protein coincubation NE *in vitro*. Prescence of minor amounts of spike amyloid fibrils during fibrin formation from fibrinogen resulted in impairment of fibrinolysis. Preliminary data shows that seeding recombinant Aβ peptides with Spike amyloid affected fibrillation kinetics. Our data propose a molecular mechanism for potential amyloidogenesis of SARS-CoV-2 S-protein in humans facilitated by endoproteolysis. The adverse effect of the generated amyloid appears to be multifaceted. The prospective of S-protein amyloidogenesis in SARS-CoV-2 Spike associated pathogenesis can be important in understanding the acute and long COVID -19 (Figure 1).

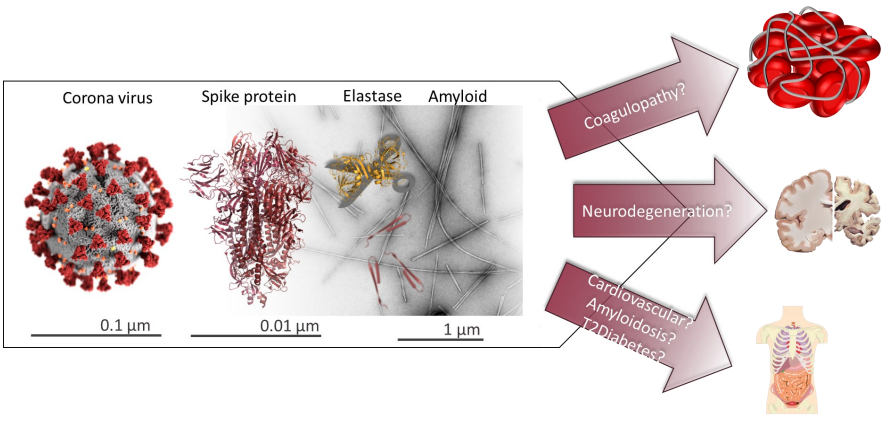

Figure 1. Proposed mechanism and possible multifaceted implications of SARS-CoV-2 amyloidogenesis

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Bri2 BRICHOS as a transport vehicle for brain delivery of proteins

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Background: The amyloid beta peptide (Aβ) forms neurotoxic oligomers that eventually form extracellular amyloid plaques in Alzheimer's disease (AD). The BRICHOS domains from human integral membrane protein 2B (Bri2) and prosurfactant protein C (proSP-C) efficiently prevent Aβ42 aggregation and neurotoxicity *in vitro* and *in vivo*. Intravenously administered recombinant human (rh) Bri2 BRICHOS passes into the brain parenchyma in WT mice and may be harnessed in managing AD.[1] For studies of kinetics and mechanisms of transport over the blood-brain barrier (BBB), *in vitro* models are being used. One model for such studies is the human cerebral microvessel endothelial cell line (hCMEC/D3), that mimics the human BBB, is reproducible and can easily be grown.[2]

Questions addressed: One major challenge to biological drugs, including antibodies, is their inherent low passage over the BBB. This study investigates if rh Bri2 BRICHOS can be used as a transport vehicle to facilitate the uptake of proteins linked to it, which could allow the development of novel therapeutic strategies. In this study we test the passage of two unrelated proteins fused to rh Bri2 BRICHOS: mCherry, a fluorescent protein derived from sea anemones, and NT*, a solubility-enhancing protein domain derived from spider silk.

Methods: hCMEC/D3 cell line was cultivated in EndoGRO-MV Complete Media, supplemented with human bFGF in a humidified atmosphere (5%CO_2) at 37°C. Cell monolayers were prepared by seeding 50,000 cells/cm^2 on collagen-coated 24-well PET membrane inserts (0.4 µm pores). The integrity of the monolayers was analyzed by crystal violet staining and FITC-dextran permeability. Permeation experiments were conducted on culture day 6 by adding BRICHOS-target protein constructs, or relevant controls to the apical side. After 24 h, the medium on the apical and basolateral sides of the monolayer were analyzed by Western blotting and the band intensities measured by ImageJ. Cell viability after the treatment was assessed by MTT and propidium iodide assays. The proteins were produced in *E. coli* and purified using immobilized metal ion chromatography and size exclusion chromatography.

Results and discussion: Rh Bri2 BRICHOS monomers show significant passage from the apical side of the monolayers to the basolateral side, ie they are transcytosed. Neither mCherry nor NT* passed through the monolayer, but the fusion constructs with rh Bri2 BRICHOS domain were transcytosed in substantial quantities, as showed in figure 1. These findings support that rh Bri2 BRICHOS can be used to transport different proteins and other macromolecules, including biologic drugs, over the BBB.

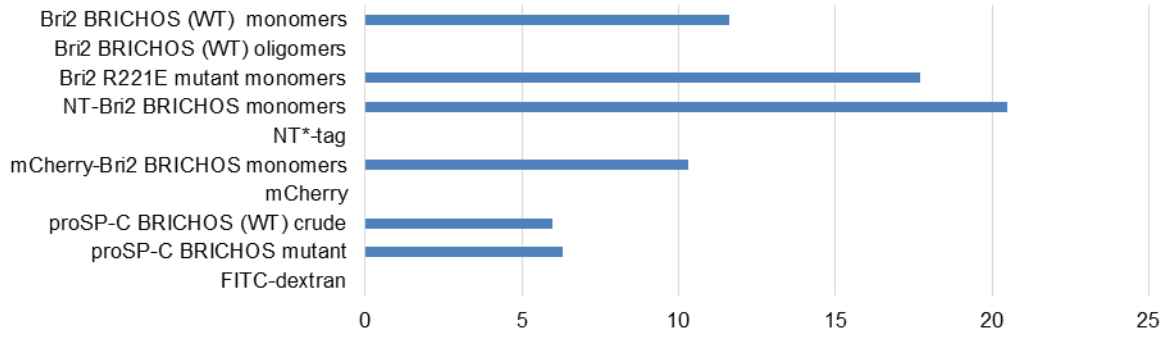

Figure 1. hCMEC/D3 monolayer permeability of different proteins and macromolecules (in percent of total amount added to the apical side).

References:
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Short truncated tau fragment 321-391 aggregates in the presence of heparin despite the lack the PHF6 epitote

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Background: Microtubule associated protein tau is the main actor of tau hypothesis of Alzheimer´s disease (AD). Tau belongs to intrinsically disordered proteins (IDPs) which do not acquire any stable secondary nor tertiary structure. Under pathological conditions of tauopathy, tau dissociates from microtubules to form insoluble filaments with disease specific fold. It was previously shown that the presence of two hexapeptide sequences (VQIxxK) can trigger the protein aggregation.[1] Recently it was shown that the AD specific fold of tau filament can be recapitulated in vitro by aggregation of truncated tau 297-391 (dGAE).[2,3]

Questions addressed: It was long believed that aggregation prone sequences were responsible for tau aggregation. It begins to be apparent that sequences in R' region that follow tau MTBR repeats play a crucial role in MT binding and potentially also in aggregation process.[4] Tau oligomers that spread the specific disease fold strain may be the pathological agent of disease and thei structural features remain still elusive.

Methods: Recombinant truncated tau proteins tau306-391, tau316-391, tau321-391 and tau326-391 were aggregated under different conditions (presence of heparin and DTT). Results of aggregation were monitored using different techniques: ThT fluorescence, DLS, AFM, FTIR and capillary electrophoresis.

Results and discussion: We have observed in vitro aggregation of several tau proteins, mainly tau 321-391 which lacks the aggregation prone sequence VQIVYK (PHF6 epitope). Early signs of oligomer formation was observed by measurements using capillary electrophoresis. The results will further widen the knowledge about pathological aggregation of tau proteins.

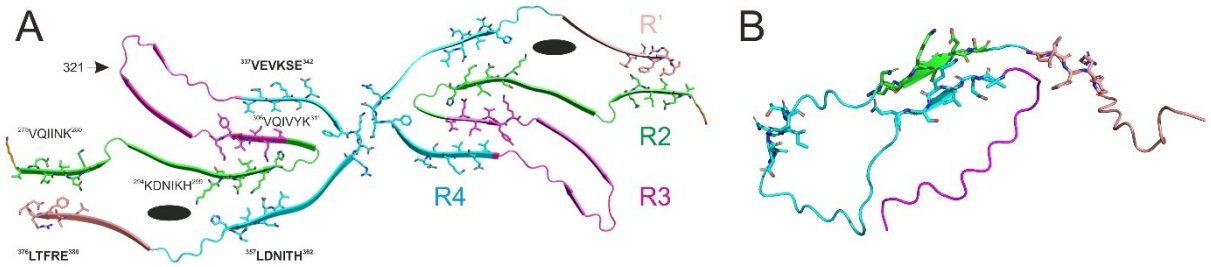

Figure 1. (A) Structure of tau filament isolated from pacient suffering with corticobasal degeneration determined by cryo-EM, with highlighted steric zipper type interfaces. **(B)** Structure of tau 321-391 predicted by I-TASSER webserver (https://zhanggroup.org/I-TASSER).

References:
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Conformational dynamics by NMR on crystals: a tool for detecting the aggregation propensity of folded and soluble proteins?

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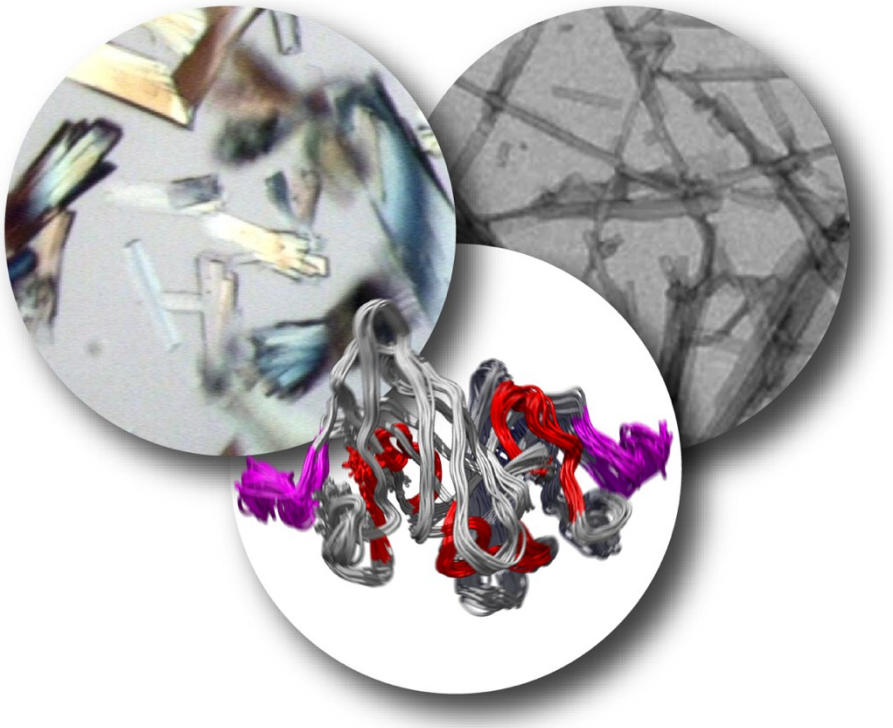
Building on a decade of continuous advances of the community, the recent development of very fast (100 kHz and above) magic-angle spinning (MAS) probes has revolutionised the field of solid-state NMR, enabling a ten-fold reduction of the required sample amounts with respect to conventional approaches, and opening the way to the application of 1H detection techniques in fully-protonated substrates.[1] Extensive and robust resonance assignments can be derived rapidly, enabling the determination of complex architectures through the detection of resolved inter-nuclear proximities.

The first part of this talk will review the strategies underlying this recent leap forward in sensitivity and resolution, describing its potential for the detailed characterisation of the structure of amyloid fibrils, a necessary step for understanding the mechanisms underlying aggregation.[2,3]

The second part will describe the new capacity of NMR in these fast MAS regime to obtain information on the dynamics of protein backbone and side-chains,[4] and show the potential of this technique for detecting 'hidden' pathological conformations of soluble aggregation-prone proteins within crystal formulations. These experiments provide a molecular explanation for the destabilisation of a native fold at the earliest steps of fibrillisation.[5,6]



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Determining Structure of S100A9 Amyloid Fibrils by Cryo-EM

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Background: Over the last decade Cryogenic electron microscopy (Cryo-EM) has emerged as an essential tool in solving large protein structures. Notably, it also allows looking into the structures of amyloid fibrils, which are insoluble protein aggregates and composed of stacked beta-sheet structures.[1] In our group, we started to adapt Cryo-EM to our needs and used it to solve the structure of S100A9 amyloid fibrils. S100A9 is a pro-inflammatory protein and is known to aggregate during Alzheimer's disease.[2] Unlike other amyloid fibrils, S100A9 contains both α-helixes and β-sheets based on the secondary structure analysis,[3] indicating it forms unique amyloid fibrils, that were not observed previously at the atomic level.

Questions addressed: Currently, there is no structural model of S100A9 amyloid fibrils. We are aiming to solve the structure of S100A9 amyloid fibrils for the first time. The structure of S100A9 fibrils would help in understanding how it aggregates and consequently would allow the development of specific drugs to inhibit aggregation. Furthermore, this model can be used as a template for solving fibril structures of other members of the S100A protein family.

Methods: 3.5 µl of S100A9 fibrils solution were applied to Quantifoil 1.2/1.3 electron microscope grid that was glow-discharged for 4 min. Grids were blotted with filter paper to remove the excess sample and plunge frozen into liquid ethane using a Vitrobot Mark IV (ThermoFisher Scientific). The cryo-EM dataset was collected on a 200 kV Glacios microscope with Falcon 3EC Direct Electron Detector and Volta Phase Plate (ThermoFisher Scientific). Particle analysis and reconstruction were done using CryoSparc[4] and Relion.[5]

Results and discussion: 1050 micrographs were recorded for the S100A9 Cryo-EM dataset and 1,36 million particles were extracted using a template obtained from manual picking. Particles were sorted into a hundred 2D classes and the ten best classes were selected for model reconstruction. The 2D indicated that fibrils consist of a dense core surrounded by lower density blobs. We were able to determine the initial model of S100A9 amyloid fibrils at 5.53 Å. The diameters of fibrils varied from 4 nm to 6 nm. In the next step, further refinement and reconstruction using Relion[5] will be performed to achieve a higher resolution model of fibrils and fit density to S100A9 residues.

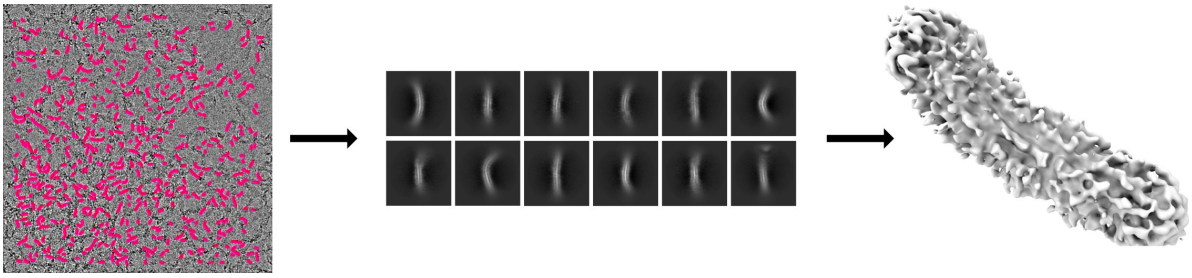

Figure 1. Analysis of S100A9 fibrils using CryoSparc.[4] Particles were picked using a filament tracker and selected best classes were used for reconstruction of the initial S100A9 amyloid fibril model.

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Ex-vivo structural biology: new insights on systemic amyloidosis

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Background: Systemic amyloidosis is a group of diseases whereby amyloid aggregation targets one or several internal organs. Amyloid deposits localize in the extracellular space and consist of cross-β misfolded protein assemblies. Among this group of diseases, Light chain amyloidosis (AL) is the most common systemic amyloidosis, and cardiac involvement is the most life-threatening form of the disease. AA amyloidosis has been found in humans and in several animal species and typically targets kidney, spleen, and liver.

Questions addressed: Structures of amyloid fibrils from ex vivo extracted material.

Methods: Recently, single particle Cryo electron microscopy (Cryo-EM) allowed a substantial leap forward in the understanding of the structural properties of amyloid fibrils. Cryo-EM can determine fibrillar structures to nearly atomic resolution not only of fibrils prepared in vitro in research labs but also of fibrils extracted ex vivo from the targeted organs of affected patients (Swuec et al. 2019, Schulte et al. 2022).

Results and discussion: Here we show three examples of Cryo-EM structures of ex vivo fibrils from the autoptic hearts of two AL patients (Swuec et al. 2019 and unpublished data) and from the kidney of a cat died of AA amyloidosis in a cat shelter (Schulte et al. 2022). Structural heterogeneity of amyloids, amyloid folding and proteolysis are discussed based on the structural data.

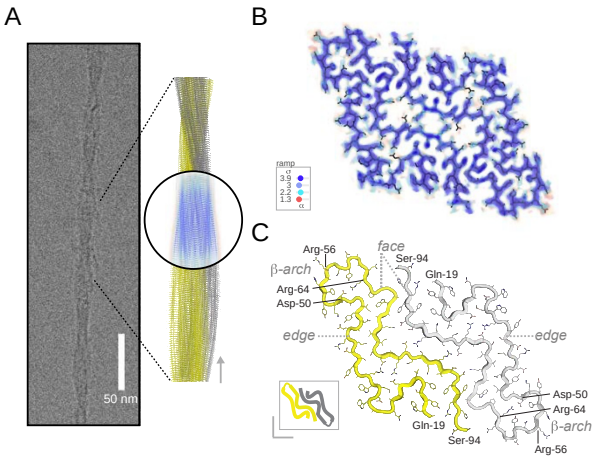

Figure 1. The 3.3 Å resolution cryo-EM structure of the cat's SAA fibril. (A) Cryo-EM image of a single straight fibril with a crossover distance in the 650-700 Å range. (B) Cross-sectional view of the map volume with contour levels according to the depicted σ-color scale. (C) The molecular model of two subunits within a single fibril layer is shown as cartoon with side chains in yellow and grey.

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Structural insights into the anti-amyloid Bri2 BRICHOS chaperone binding to Alzheimer's associated amyloid-β fibrils

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Background: In Alzheimer's disease (AD), the most prevalent form of dementia, the pathogenesis is closely associated with fibril formation of the amyloid-β peptide (Aβ). So far, no disease-modifying drug is available, making new approaches necessary where molecular chaperones are promising examples. In particular, the chaperone domain BRICHOS from Bri2 has been shown to specifically target and decrease toxicity associated with amyloid generation and it passes the blood-brain barrier.

Questions addressed: So far no molecular structure is available for Bri2 BRICHOS and, in general, the structural basis for molecular chaperone-amyloid fibril interactions is still an open question.

Methods: We use a combined approach of solution NMR and molecular dynamics simulation to investigate the secondary structure propensities of Bri2 BRICHOS and characterize its dynamics in solution. To study the structure of Aβ42 fibrils co-incubated with Bri2 BRICHOS we apply EM analysis and solid-state NMR, combined with DNP enhancement.

Results and discussion: Here, we report detailed insights into the structure and dynamics of Bri2 BRICHOS in solution and its binding to Aβ42 fibrils *in vitro*. Bri2 BRICHOS is highly dynamic in solution, where we characterized the protein dynamics and secondary structure propensity of Bri2 BRICHOS in solution. Further, our EM analysis of Aβ42 fibrils co-incubated with Bri2 BRICHOS revealed that the fibril diameter is decrease to about half size in the presence of BRICHOS. Applying solid-state NMR, combined with DNP enhancement, we could detect BRICHOS and identify a specific modulation site of Bri2 BRICHOS on Aβ42 fibrils.

These results facilitate a comprehensive understanding of chaperone-modulated Aβ aggregation, which might be helpful to design novel ways to utilize BRICHOS in AD treatments.

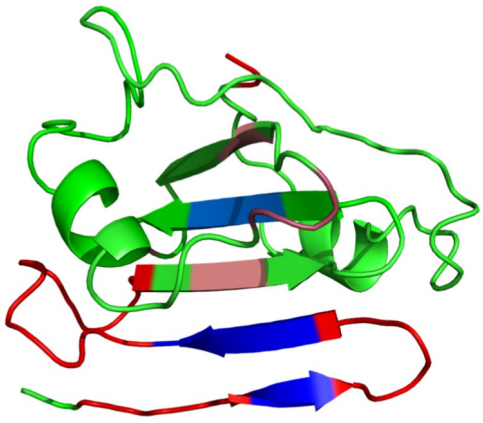

Figure 1. Predicted structure of monomeric Bri2 BRICHOS in solution where visible residues in solution NMR spectra are marked with dark red color for loop residues and dark blue color for β-sheet residues (as assigned from the chemical shifts by TalosN[1]) and light colors for weak assignments.

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Kinetics-Based Drug Discovery for Alzheimer's Disease

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The phenomenon of protein misfolding and aggregation is associated with a wide range of human disorders, including Alzheimer's and Parkinson's diseases. A central role in these conditions is played by protein misfolded oligomers, which are among the most cytotoxic products resulting from the process of protein aggregation. It has been very challenging, however, to target these oligomers with therapeutic compounds, because of their dynamic and transient nature. To overcome this problem, I will describe a kinetic-based approach, which enables the discovery and systematic optimization of compounds that reduce the number of oligomers produced during an aggregation reaction. I will illustrate this strategy for the amyloid beta peptide, which is closely linked to Alzheimer's disease. As this strategy is general, it can be applied to oligomers of any protein in drug discovery programmes.

Autoxidation Increases Anti-Amyloid Characteristics of Flavones

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Zvinys Gediminas, Smirnovas Vytautas

Institute of Biotechnology, Life Sciences Center, Vilnius University, Vilnius, Lithuania

Background: Protein aggregation into beta-sheet rich structured aggregates is associated with various neurodegenerative disorders such as Alzheimer's (AD) and Parkinson's (PD) disease. AD alone was recognized to be the most common neurological condition affecting over 35 million people worldwide and is expected to rise up to 76 million by 2030. The increase in cases of those who suffer from neurodegenerative diseases creates a need for a potent anti-amyloid pharmaceutical. It has been described on multiple occasions that flavones, a group of naturally occurring antioxidants, influence the aggregation of different amyloidogenic proteins, including amyloid-beta. Various flavones also belong to the group of acetylcholinesterase inhibitors, that are shown to possess positive characteristics in the treatment of AD symptoms. However, due to the autoxidation of flavones at neutral pH, it is uncertain if the effective inhibitor is the initial molecule or a product of this reaction, as many anti-amyloid assays attempt to mimic physiological conditions.

Questions addressed: In this research, we tried to understand the impact of 64 different hydroxy-flavones and their oxidation products on the amyloid aggregation process. Further, we questioned which newly-formed molecules are responsible for inhibiting amyloid aggregation in the oxidation mixture; how they impact the protein aggregation, aggregate morphology, and toxicity to cells.

Methods: In this work, we test the aggregation-inhibition properties of flavones before and after they are oxidized. The flavone oxidation process was tracked by measuring the absorbance spectrum change over time. The protein aggregation kinetics were followed by measuring the amyloidophilic dye thioflavin-T (ThT) fluorescence intensity change. The analysis of the most potent inhibitor was done using HPLC. The inhibitor was separated into two fractions that contained low and high molecular weight molecules. The components were tested using NMR and MALDI-TOF. Atomic force microscopy was employed to image the aggregates formed with different inhibitor concentrations. MTT assay was used to understand how the inhibiting molecules change the toxicity to SHSY-5Y cells.

Results and discussion: We show that the tendency to autoxidize is related to the positions of the flavone hydroxyl groups and that flavones, which undergo autoxidation, have a far greater potency at inhibiting the aggregation of both the disease-related amyloid-beta, as well as a model amyloidogenic protein - insulin. Oxidized 2'3'-dihydroxyflavone was the most potent inhibitor affecting both insulin and amyloid-beta. Further studies with oxidized 2'3'-dihydroxyflavone showed that the polymeric fraction (large molecular weight components) in the sample are the key molecules affecting amyloid-beta and alpha-synuclein aggregation and their toxicity to cells.

Structural basis for the inhibition of IAPP fibril formation by the co-chaperonin prefoldin

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Background: Chaperones, as modulators of protein conformational states, are key cellular actors to prevent the accumulation of fibrillar aggregates. Here, we integrated kinetic investigations with structural studies to elucidate how the ubiquitous co-chaperonin prefoldin (PFD) inhibits diabetes associated islet amyloid polypeptide (IAPP) fibril formation.[1]

Questions addressed: IAPP aggregation kinetics and aggregates morphology in absence and presence of PFD; interaction between monomeric IAPP and PFD: identification of binding sites and dissociation constant; interaction sites of PFD on IAPP fibril surface and ends; cell viability in presence of IAPP aggregates depending on PFD amount present.

Methods: fluorescence-based kinetic assays (ThT assays), nuclear magnetic resonance (NMR), atomic force microscopy (AFM), electron microscopy (EM), cryo-EM, biolayer interferometry (BLI), cell viability assay (MTT), modeling (docking).

Results and discussion: We demonstrated that both human and archaeal PFD interfere similarly with IAPP fibril elongation and secondary nucleation pathways. Using archaeal PFD model, we combined NMR spectroscopy with EM to reveal that the inhibition of fibril formation is mediated by the binding of PFD's coiled-coil helices to the flexible IAPP N-terminal segment accessible on the fibril surface and fibril ends. AFM showed that binding of PFD to IAPP leads to the formation of lower amounts of aggregates, composed of shorter fibrils, clustered together. Linking structural models with observed fibrillation inhibition processes opens perspectives for understanding the interference between natural chaperones and formation of disease-associated amyloids.[1]

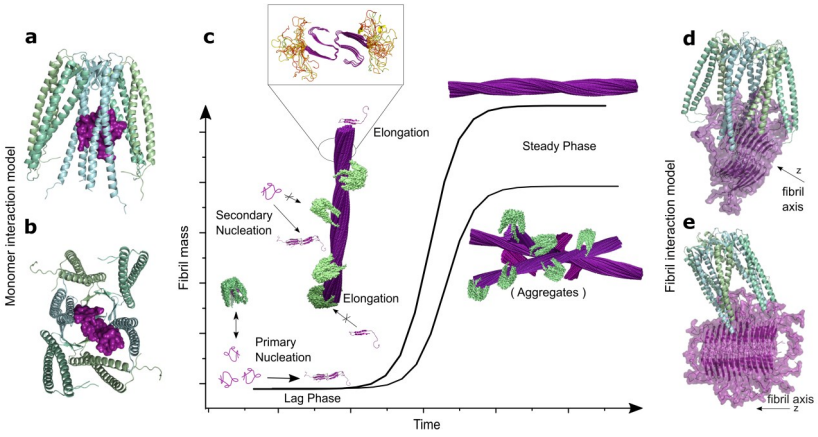

Figure 1. Structural schematic model of inhibition of IAPP fibril formation by PFD. a, **b** Docking models of the complex between monomeric IAPP (PDB: 2L86) and PhPFD (PDB: 2ZDI) based on NMR derived interaction information. **c** Inhibition of secondary nucleation and elongation results from coverage of fibril surface and ends by PFD. The presence of PFD leads to a decreased steady phase fibril mass associated with formation of less aggregates with an altered morphology. The inset zoom represents a model of IAPP fibril structure with unfolded residues 1–12 in yellow and the structured fibril core in purple (residues 13–37). **d**, **e** present docking models of PhPFD (PDB: 2ZDI) on IAPP fibril (PDB: 6Y1A) surface and extremities, respectively, integrating structural information obtained by NMR and EM; the black arrows indicate the fibril axis with the tips pointing towards the fibril ends.[1]

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A super-efficient anti-amyloid chaperone domain BRICHOS in inhibition of amyloid fibril formation

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Background: Formation of fibrillar amyloid deposits is linked to devastating amyloid diseases, including neurodegenerative disorders like Alzheimer's disease. The anti-amyloid chaperone domain, BRICHOS, ~100 aa in size, is present in proteins that all contain a region with high amyloid-forming propensity. The biological role of this chaperone domain is to protect the native "client peptide" from forming amyloid, while, recently, recombinant BRICHOS domains can also inhibit amyloid fibril formation of "non-client peptides" linked to human diseases, holding great pharmaceutic potential. The BRICHOS domain is produced by metazoan species with rather low amino acid sequence similarities and could be grouped into 13 unrelated families, while the so far studied three BRICHOS domains have displayed different chaperone activities.

Questions addressed: We hypothesized that in nature there are BRICHOS domains holding super-efficient capacities in preventing amyloid formation.

Methods: The BRICHOS domains were recombinantly expressed and characterized by size exclusion chromatography, circular dichroism (CD), anti-citrate synthase (CS) assay, thioflavin T (ThT) assay, kinetics analysis, and transmission electron microscopy.

Results and discussion: One of the BRICHOS families is gastrokine 1 (GKN1), which is associated with gastric cancer and specifically expressed in the stomach. The human GKN1 is a secretory protein with 185 aa in size, harboring a BRICHOS domain and a C-terminal amyloid-prone region. The NT*-GKN1 BRICHOS mainly forms monomers, dimers, and a tiny number of oligomers, where NT* is a solubility tag. While the GKN1 BRICHOS monomer is not stable, the pure dimer and oligomer were successfully isolated, which are similar to BRICHOS domains from other families prepared previously in terms of the secondary structure indicated by CD. The anti-CS assay revealed that GKN1 BRICHOS doesn't obviously suppress thermo-induced amorphous aggregation. Surprisingly, the results of the ThT assay showed that the GKN1 BRICHOS dimer is extremely efficient in inhibiting amyloid fibril formation, suppressing Aβ42 fibril formation completely for ~25 h (lag phase), which has never been seen for other so far studied BRICHOS domains. Further kinetics analyses indicated that GKN1 BRICHOS inhibits Aβ42 fibril formation by affecting both primary nucleation and secondary nucleation, different from already studied BRICHOS domains. This study provides an efficient BRICHOS domain, which might be able to be used for counteracting amyloid disease.

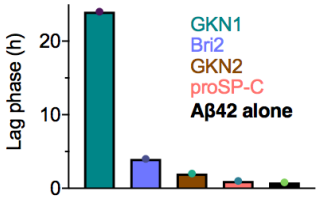

Figure 1. Lag phase duration of 3 μM Aβ42 fibrillization in the presence of amyloid chaperone domains (molar ratio 1:1) from GKN1[(unpublished)], Bri2,[1] GKN2[(unpublished)] and proSP-C,[2] respectively.

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Reversable Crystallization of Amyloid Proteins

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The self-assembly of amyloid protein is associated with both the pathology mechanics studies as well as the fabrication of functional materials in the fields of materials science and nanotechnology. There is a genuine need to understand the hierarchical mechanism of amyloid formation with special care about its kinetics and thermodynamics to control these events. There is a special interest in the transition of normal proteins into the amyloidal architectures. Commonly the hierarchical process of amyloid self-assembly has been shown for fibrillation. The highly ordered amyloid crystal arrangement can also be achieved by different assembling strategies. In this talk, the assembling strategies will be introduced to control peptide growth from fibrillation to crystallization for both the common cross-β structures and a novel cross-α amyloid-like structures. Such crystallization process can lead to grow into macroscopic crystals. By carefully investigation the structural characteristics and formation mechanism at molecular level, one can control the dynamic process of peptide self-assembly. Therefore, the reversable disassembly process of crystal controlled have been achieve successfully by interrupting noncovalent interactions. The reversible assembly and disassembly of crystals benefit not only the understanding of dynamic process of pathology peptide self-assembly but also creating novel function biomaterials.

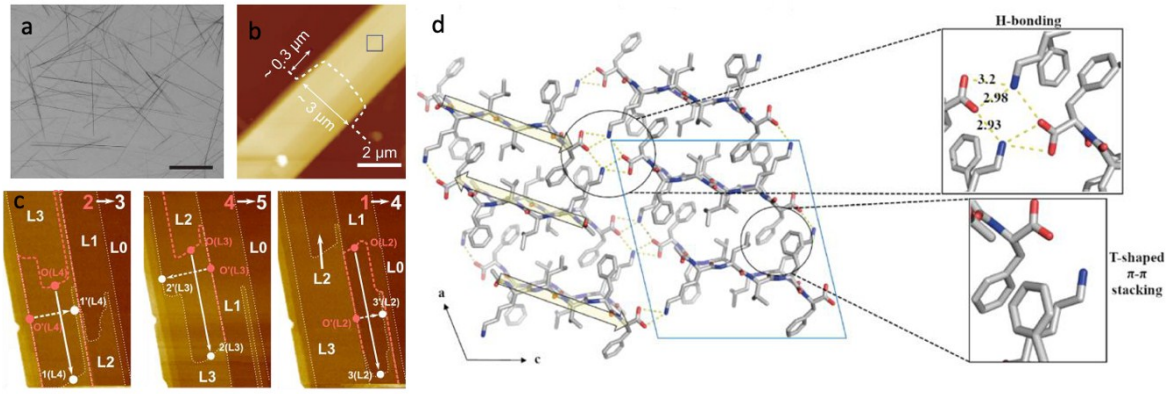

Figure 1. Optical microscopic images of Ac-KLVFF crystals (Scale bar: 200 μm.), High resolution AFM image of single crystal. In situ Dynamic AFM Analysis of disassembly process of crystal, Atomic structure and electrostatic interactions of Ac-KLVFF peptide crystal. (Molecular structure projected along b-axis, showing the hydrogen bonds between 2D lamella and T-shaped π-π stacking)

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Fluorescently guided optical photothermal infrared microspectroscopy for detection of structurally altered amyloid proteins in cells and tissues at a subcellular level

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Background: One of the key pathological features of many neuropathological diseases is related to the aggregation of misfolded proteins, so-called amyloid oligomers. Immunofluorescence can be used to label amyloids in tissue but cannot perform in-situ chemical structural analysis to analyze the misfolded proteins, while Infrared (IR) spectroscopy can readily detect conformational changes associated with amyloid formation via specific infrared absorption bands associated with β-sheets within the Amide I protein absorption band. However, conventional IR spectroscopy has spatial resolution limited to the scale of ~10 microns, making it too coarse for sub-cellular analysis. To date, no instruments have been unavailable to perform IR spectroscopy on fluorescently labeled biological samples with submicron spatial resolution. Thus it is very challenging to address questions related toxicity of amyloid structures.

Methods: We applied fluorescently guided optical photothermal infrared (OPTIR) microscopy to locate microglia cells in brain tissue and examine β-sheet structures. OPTIR is a novel form of infrared spectroscopy that can achieve a spatial resolution of <500 nm. We used a novel instrumental setup that combines OPTIR with an epifluorescent imaging module (EF-OPTIR) to allow IR spectroscopic analysis of fluorescently labeled regions in cells and tissues. For the proof of concept experiment, microglia cells and amyloid proteins were immunofluorescently labeled with specific antibodies to enable fluorescence imaging to identify and select specific locations of the cells in mouse brain tissue, model of AD. We then used OPTIR to take infrared spectra at the selected fluorescently labeled locations to perform molecular structure analysis of microglia cells surrounding amyloid plaques regions as compared to microglial cells in amyloid-free tissue. We have demonstrated the capability of OPTIR microscopy to locate specific cells and amyloid proteins and assess amyloid structures with sub-micron spatial resolution.

Results and discussion: We have demonstrated the potential of applying infrared spectroscopy for neuroscience by increasing both the spatial resolution and the specificity of chemical structure analysis in tissues and cells, thus overcoming the current limitations of conventional fluorescence and infrared microspectroscopy. We believe that our new approach will be helpful in studies of the role of the brain environment in the formation of structurally altered protein aggregates which are highly relevant to many neurodegenerative diseases.

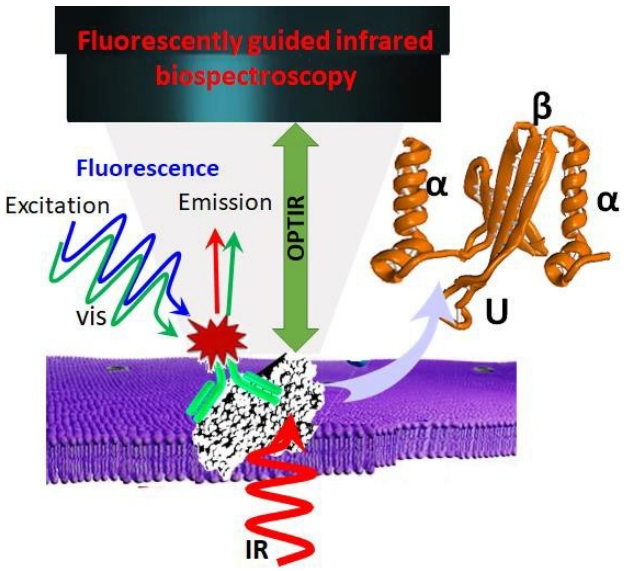

Figure 1. Schematic diagram of the working principle and lateral resolution for fluorescently guided optical photothermal infrared microspectroscopy.

Structure and interactions of amyloid-β peptide aggregates unraveled by novel infrared spectroscopy approaches

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Background: The most common neurodegenerative disease is Alzheimer's disease, in which the amyloid-β (Aβ) peptide aggregates to amyloid fibers that accumulate in plaques in the human brain.

Questions addressed: Our work sheds light on two key aspects of the disease that are still unclear: (i) the structure of Aβ oligomers in aqueous solution and (ii) interactions of Aβ with other peptides.

Methods: We exploit the particular advantages of isotope-edited infrared (IR) spectroscopy using both uniform and site-specific 13C-labeling.

Results and discussion: Our results confirm a hairpin structure of individual peptides in the oligomers as they indicate a building block of two adjacent strands for Aβ40 and of two or more adjacent strands for Aβ42.[1] For more detailed structural models, we use ^{13}C–labeling of specific amide groups in the backbone to identify intra– and intermolecular contacts similar to solid state nuclear magnetic resonance. First results indicate that the β-sheets of oligomers are composed of different residues in oligomers of different sizes (Vosough & Barth, manuscript in preparation).

To study Aβ's interaction with other peptides, we use uniform ^{13}C–labeling of one of the interaction partners. We found that Aβ40 and Aβ42 form mixed β-sheets in oligomers.[2] With IR nanospectroscopy, we were able to identify both interaction partners in nanoscale images of the IR absorption. Our results, shown in Figure 1, indicate that an anti-amyloid peptide dissolves or coats Aβ fibers.

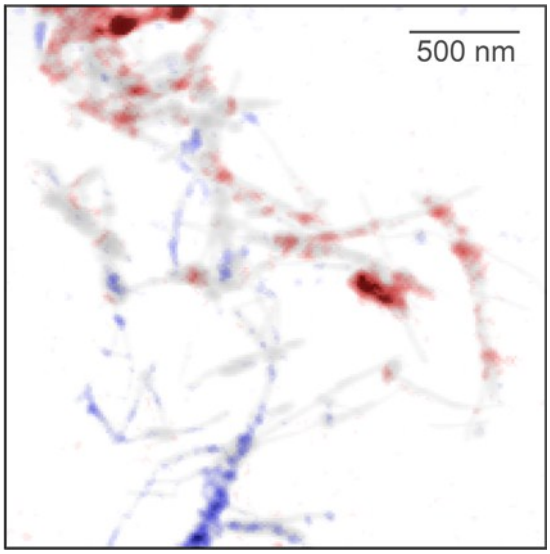

Figure 1. Nanoscale imaging of ^{13}C-Aβ40 and ^{12}C-anti-amyloid peptide. The height of the thinnest fibrils is 2.5 nm. Grey: height profile, red: mostly Aβ40, blue: mostly anti-amyloid peptide. Blue and red areas were ob-tained from two images of the IR ab-sor-bance taken at wave-numbers where the absorption is either strong for the ^{13}C- or for the ^{12}C-peptide. The images were subtracted and the resulting difference image overlaid with the AFM height image. The result clearly reveals regions of mostly Aβ40 (red), mostly anti-amyloid peptide (blue), and both (grey, absorbance difference ≈ 0). The fibers contain either the anti-amyloid peptide or both peptides, but not predominantly Aβ40, although Aβ40 alone formed fibrils. Controls of either purely unlabeled or purely labelled peptide showed the expected stronger absorbance at the wavenumber characteristic for the used isotope.

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A fluorescent fusion protein as a structural probe to monitor Aβ-amyloid fibril polymorphism

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Background: Alzheimer's disease (AD) is the most common neurodegenerative disease worldwide. AD is associated with misfolding and aggregation of Aβ and tau proteins into amyloid fibrils in the brain. Various *in vivo* and *in vitro* studies have established the link between Aβ amyloid fibril structural polymorphism and AD progression. In this context, it is crucial to study and understand real-time Aβ aggregation in animal models and *in vitro.*

Questions addressed: Will labeling Aβ with fluorescent protein help in studying real-time fibril dynamics and polymorphism in *in vitro* and organisms?

Methods: We made fusion constructs in which the N-terminal of Aβ1-42 is labeled with mNeon Green (mNG). mNG-Aβ1-42 was expressed in bacteria and purified as per standard protocol. Aggregation kinetics was monitored using the ThT assay, and aggregates were further characterized using transmission electron microscopy (TEM). To study Aβ1-42 amyloid fibril polymorphism *in vivo*, transgenic Drosophila with gene insertion of UAS-mNG-Aβ1-42, UAS-mNG, were generated. Flies co-expressing both mNG-Aβ1-42 and Aβ1-42 were also made.

Results and discussion: *In vitro* aggregation kinetics data suggest that mNG-Aβ1-42 delays Aβ1-42 fibrillation. Hyperspectral imaging shows that mNG-Aβ1-42 gets incorporated in Aβ1-42 fibrils *in vitro*. Data on flies co-expressing mNG-Aβ1-42 together with Aβ1-42 showed partial attenuation of Aβ1-42 induced toxicity. The X-34 amyloid dye was used to stain the whole brain and cryosections from these flies. We observed mNG positive amyloid aggregates of Aβ1-42 corroborating the *in vitro* results of incorporation of mNG-Aβ1-42 into Aβ1-42 fibrils *in vivo*. Spectral data suggest that X-34 and mNG are excellent donor-acceptor pairs for FRET analysis. We used confocal microscopy with FLIM to study aggregates formed in different cell types. FRET analysis of co-aggregated mNG-Aβ1-42 with Aβ1-42 fibrils formed from two distinct subtypes of cells (glia versus neurons) revealed easily quantifiable FRET efficiency between X-34 and mNG. FRET efficiency in glial aggregates compared to neuronal aggregates depended on genotype and age. Our FLIM data suggest that different Aβ1-42 fibril polymorphs are produced in different cell types, corroborating our group's previous results (Jonsson 2018). This new reporter protein allows us to do in-depth (low-resolution) structure analysis of temporal and genetic fibril structural polymorphism in real-time *in vivo*.

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Inhibition of islet amyloid formation in functional insulin-producing islet-like cell clusters

Gunilla T Westermark, Jing Cen, Joey Lau Börjesson

Islet amyloid, described in early 1900,[1] was biochemically characterized in 1986 and found to be a novel polypeptide hormone named IAPP.[2] Initially, islet amyloid was believed to be restricted to type 2 diabetes, but further studies revealed IAPP deposits in connection with type 1 diabetes (T1D), pancreatic cancer, transplanted islets, and cystic fibrosis. All four conditions are linked to β-cell stress known to stimulate hormone synthesis, including IAPP, a prerequisite for islet amyloid formation.

Transplantation of human islets has been explored as a treatment for T1D for more than 30 years, but with limited success. Instead, the hope has turned to generate new insulin-producing β-cells from human pluripotent stem cells. Human pluripotent stem cells (hPSC) can be differentiated via a 7-step protocol into functional insulin-producing islet-like cell clusters (ICCs).[3] However, in a situation where ICCs were exposed to stress, IAPP amyloid developed. Due to IAPP's high propensity to misfold, it has been suggested that an endogenous inhibitor should exist. Bri2 is a multi-domain protein, and recently we showed that the BRICHOS domain of Bri2 is sufficient to block IAPP aggregation in vitro and ameliorate cell death linked to metabolic stress.[4]

We have investigated if BRICHOS can act as an inhibitor and prevent the amyloid formation in ICCs exposed to metabolic stress. ICCs were transduced with adenovirus (Ad-BRICHOS) and cultured in 20 mM glucose. Clusters recovered after 9 days of culture were stained for amyloid. The amyloid load was shown to be decreased in clusters transduced with Ad-BRICHOS as compared to the amyloid load present in untransduced clusters. IAPP amyloid formation is dependent on functioning β-cells expressing the hormone. To ensure hormone synthesis, we analyzed glucose-stimulated insulin release in Ad-BRICHOS transduced clusters. Secreted insulin levels were similar to levels detected in control clusters cultured in 5.6 mM glucose.

Conclusion: The development of IAPP amyloid is a severe drawback for the usability of stem-cell-derived clusters as treatment in type 1 diabetes. The observed amyloid reduction in clusters over-expressing Ad-BRICHOS opens new opportunities to prevent IAPP amyloid formation in these cells.

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Probing α-Synuclein conformational space by acetyllysine-mimicking mutations

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Aggregation and intracellular accumulation of α-synuclein (αSyn) is a hallmark of several diseases, including Parkinson's, multiple system atrophy (MSA), or Lewy body dementia.[1] Interestingly, the spontaneous aggregation of αSyn is slow under the physiological solution conditions due the high energy barrier of homogeneous nucleation.[2] The aggregation rate is accelerated by the presence of physical or biological interfaces, most notably lipid membranes and preformed αSyn fibrils (seeds).[3] Under specific conditions, αSyn can undergo liquid-liquid phase separation (LLPS) by spontaneously separating into a dilute and dense phase.[4] Although initially reversible, the high protein concentration of the dense phase strongly favors the aggregation of αSyn into amyloid fibrils.[5] This immense conformational plasticity of αSyn is further modulated by familiar mutations, environmental conditions, and posttranslational modifications (PTMs), which have all been implicated to play important roles in disease pathology.[6] One of the PTMs that influence aggregation, lipid binding, and neurotoxicity is acetylation of lysine residues.[7] The 140-residue long αSyn contains 15 lysines distributed across the sequence as parts of the seven imperfect repeats of the N-terminal domain, in the non-amyloid component domain (NAC), and at the C-terminal domain. Their mutagenesis to the acetyllysine-mimicking residue glutamine therefore represents perfect tool for probing αSyn conformational space. Here, we explore how individual KQ mutations, and their combination affect the seeding properties of αSyn, structure and stability of amyloid fibrils, and how they influence the phase separation capacity of αSyn. By systematically removing positive charges from αSyn sequence we probe the role of electrostatic interactions during conformational transitions of αSyn under different conditions. Using combination of KQ mutagenesis, small scale purification, and high-throughput biophysical assays we aim to gain important insights into the sequence determinants of αSyn conformational changes and how their regulation by acetylation as a biologically relevant PTM.

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Stability of α-synuclein fibrils in different conditions; a path to distinguish different fibril species in different synucleopathies

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It is becoming more and more evident that amyloid fibrils from Parkinson's disease (PD) and multiple system atrophy (MSA) are different in structure and stability, even though they are formed from the same protein, alpha-synuclein (α-syn). Similarly, different α-syn fibril structures are observed in *in vitro* aggregation studies where different ionic strength, pHs, small molecules, and lipids induce different fibril strains. These findings suggest that the different physico-chemical conditions in different brain regions are the reason for the different fibril strains in PD and MSA in dopaminergic neurons and oligodendrocytic glial cells, respectively. This difference can be exploited to distinguish the diseases in a biochemical/ biophysical assay. While there are several α-syn-RT-QuIC or PMCA assays for distinguishing these diseases using assays of aggregation kinetics, there is no comprehensive study on the thermodynamic stability of the fibrils. PMCA or RT-QuIC relies on amplifying aggregates in CSF, blood, or nasal brush samples, which are transferred to a tube test with a different solution condition, whereas this treatment can change fibril structure and affect the seeding properties in the new condition. Here, we study different ionic strengths (0, 150, and 1000 mM NaCl) and pHs (pH 5.5 and 7.4) to form α-Syn fibrils and then investigate the thermodynamic stability (through chemical depolymerization with urea) and the kinetics of seeding and cross-seeding. Understanding the stabilities of different fibrils under different solution conditions will help us to improve our diagnostic abilities for PD and MSA.

Understanding the molecular structure transitions and formation of recombinant beta-lactoglobulin fibrils using solid-state NMR spectroscopy

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Background The whey protein β-lactoglobulin is known to form amyloid-like fibrils under certain conditions. Today this is one of the most frequently used model systems for developing protein nanofibrils (PNFs) based materials[1]. Recently, the structural study of the amyloidogenic peptide segment of β-lactoglobulin (β-LG$_{11–20}$) has disclosed its molecular structural features as the parallel β-sheets orientation and cross-beta packing preferences[2].

Questions addressed: However, details about the intramolecular structures and hierarchy of fibrils organization remain not entirely understood.

Methods: Here, we use the extended version of the synthetic peptide fragment composed of 26 residues (β-LG$_{8-33}$) with a selected labeling scheme of amino acids 13C and 15N to acquire tertiary and quaternary structural information of PNFs using solid-state NMR. Additionally, recombinantly expressed and purified β-lactoglobulin 13C-15N labeled is submitted to fibril formation and analyzed using AFM and solid-state NMR. The Solid-state NMR analysis of selectively labeled β-LG$_{8-33}$ peptide and full β-lactoglobulin fibrils will give us insights into the PNFs mechanical and structure-function relationship.

Results and discussion: Eventually, structural models will be derived based on the NMR data.

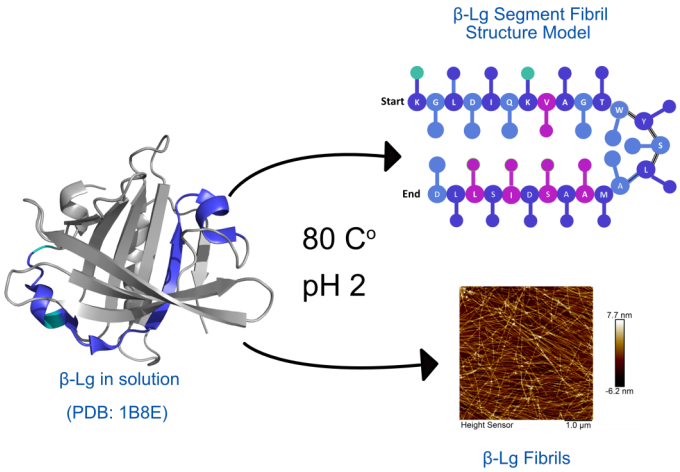

Figure 1. β-lactoglobulin thermal and pH aggregation, generates amyloid-like protein nanofibrils.

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Direct Observation of Secondary Nucleation Along Fibril Surfaces of Amyloid b 42

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Background: Alzheimer's disease is a neurodegenerative condition which involves heavy neuronal cell death in the brains of affected individuals. It is now established that the oligomers formed during the aggregation process of the amyloid β peptide 42 (Aβ42) are responsible for this neurotoxicity. The aggregation of Aβ42 involves a series of microscopic steps, of which secondary nucleation leads to the generation of the largest amount of oligomers. Secondary nucleation involves the formation of new aggregates from monomer on fibril surfaces which act as catalysts. The molecular mechanism of secondary nucleation is not yet understood, but is crucial in developing a targeted cure.

Questions addressed: We directly observe secondary nucleation using 2-color dSTORM, and ask whether secondary nucleation involves an underlying templating mechanism.

Methods: Direct Stochastic Optical Reconstruction Microscopy (dSTORM), Thioflavin T fluorescence assays, seeded aggregation kinetics, cryoTEM.

Results and discussion: Here, we study self-seeded aggregation reactions of WT Aβ42 using direct stochastic optical reconstruction microscopy (dSTORM) and separate fluorophores in seed fibrils and monomers. We compare this with cross-seeded reactions of WT Aβ42 monomer with mutant Aβ42 fibrils that do not catalyze the nucleation of WT monomers, to study whether monomer interaction is observable both with fibrils that catalyze nucleation and those that don't. The dSTORM experiments show that self-seeded aggregation proceeds faster than non-seeded reactions because the fibrils act as catalysts. The monomers are found to grow into relatively large aggregates on fibril surfaces along the length of fibrils before detaching as individual aggregates in solution. In contrast, cross-seeded aggregation progresses more slowly, similar to non-seeded reactions. While the monomers are observed by dSTORM to interact with non-cognate fibril surfaces, we fail to notice any nucleation on such fibril surfaces. The only process noticed is growth at fibrillar ends, i.e. elongation.

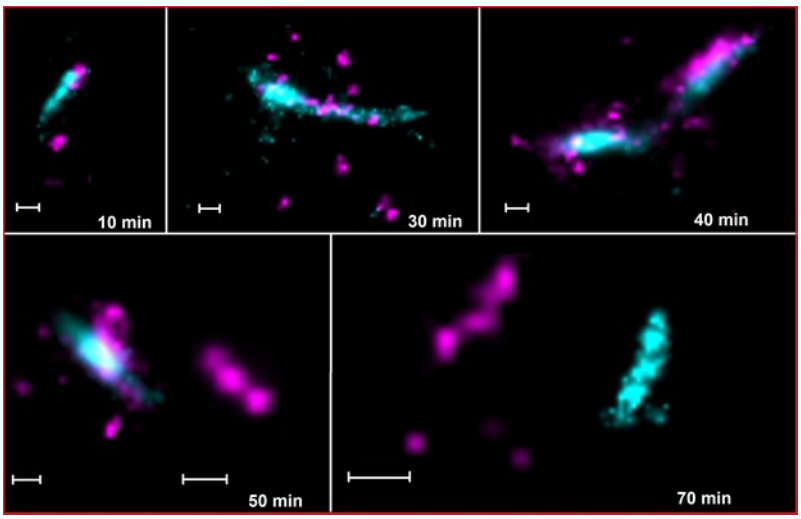

Figure 1. Self-seeded aggregation reaction of WT Aβ42 as followed by dSTORM. WT monomers (fuchsia) are labelled with Alexa647, and pre-formed WT fibrils (seeds, cyan) are labelled with Alexa488.

The impact of α-synuclein on the aggregation of Superoxide dismutase-1

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Background: Amyotrophic lateral sclerosis (ALS) is a neurodegenerative disorder that is linked to mutations of a gene encoding antioxidant enzyme Superoxide dismutase-1 (SOD1) leading to a misfolding of its molecules and their aggregation in motor neurons within the central nervous system. The formation of amyloid fibrils depends on various factors such as pH, ionic strength or the presence of other amyloidogenic proteins. α-synuclein (ASyn) is one of such proteins that form amyloid structures during Parkinson's disease and is known to interact with β-amyloid peptide and Tau protein, related to Alzheimer's disease.[1,2]

Questions addressed: Since the incidence of ALS is increasing worldwide, a deeper insight into amyloid aggregation process is very important. Researches are focused on the factors that influence amyloid fibril formation of pathogenic SOD1 molecules and may determine the change of ALS symptoms and progression rate. We aim to analyze the impact of ASyn molecules on the SOD1 aggregation process.

Methods: Recombinant C-terminally his-tagged SOD1 protein was cloned in pET303 vector, expressed in *E.coli* BL21(DE3) strain and purified using nickel ion affinity chromatography. Aggregation experiments were carried out with 200 μM SOD1 monomer and a range of monomeric and aggregated ASyn concentrations in 10 mM potassium phosphate, 0.5 M GuHCl, 5 mM DTT, pH 7.4 buffer. The monitoring of aggregation kinetics was performed using Thioflavin T fluorescence assay. The morphology and secondary structures of resulting SOD1 aggregates were determined by using atomic force microscopy and Fourier-transform infrared spectroscopy.

Results and discussion: In this work, we examined how ASyn molecules affect SOD1 aggregation kinetics, using a range of ASyn concentrations. We observed that both, ASyn monomers and aggregates, accelerated the SOD1 aggregation process by reducing the aggregation lag times. Despite altering aggregation kinetics, ASyn molecules did not have a significant influence on the morphology and the secondary structures of resulting SOD1 amyloid fibrils.

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Structural Variability of Prion Protein Amyloid Fibrils Under Different Agitation Conditions

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Background: Amyloidogenic peptides and proteins have a property to convert from their native functional states into fibrillar amyloid aggregates. This property is associated with neurodegenerative disorders, such as Alzheimer's or Parkinson's diseases, as well as prionopathies.[1] It has been observed that the environment conditions in which amyloid aggregation takes place have an important effect on fibril polymorphism. Moreover, amyloid aggregates have recently been shown to have the property to adopt more than one different conformation under the same environmental conditions.[2] One example of such environmental conditions, whose effect on amyloid structural variability is not fully understood, is agitation.

Questions addressed: Since there are countless studies employing different types and intensities of sample agitation, this environmental factor requires additional investigation. In this work, we examined the effect of three different agitation conditions on the aggregation kinetics of mouse prion protein fragment 89-230 (MoPrP) and analyzed the secondary structure of the resulting fibrils.

Methods: MoPrP 89-230 was purified by immobilized nickel ion affinity chromatography. Protein samples were incubated under three agitation conditions (200, 400 and 600 RPM) at 37°C, under denaturing conditions (2 M guanidinium hydrochloride, 50 mM sodium phosphate, pH 6.0). The kinetics of aggregation were determined by recording the fluorescence intensity of the amyloidophilic dye thioflavin-T (ThT). The secondary structure of fibrils was determined by analyzing each sample's FTIR spectra. For direct comparison, all spectra were normalized to the same area of amide I/I' band (1700–1595 cm^{-1}).

Results and discussion: The average kinetic parameters for each agitation condition (lag time and apparent fibril elongation rate) were calculated and these parameters were compared between each other. Interestingly, despite such large variations in agitation intensity, there were no significant differences between the process lag time values. The apparent rate of elongation, however, was significantly different between all three conditions. Surprisingly, the rate was highest at 400 RPM, rather than the expected 600 RPM condition. These results suggest that the intensity of agitation has a minimal influence on primary nuclei formation and that the rate of elongation does to scale with the level of agitation. In all three cases, a diverse collection of secondary structures (at least three structure types) were observed, with the highest variability detected under 200 RPM agitation conditions.

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Towards an Understanding of Aggregation Tendencies and Structural Characteristics of Amyloidogenic S100A8 and S100A9 Proteins

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Background: S100A8 and S100A9 are members of the S100 calcium-binding protein family, which has a wide range of functional diversity, implying a significant role in pathologies. Both S100A8 and S100A9 can be heavily upregulated in Alzheimer's disease patient's brain tissues[1] and tend to form amyloidogenic fibrils.[2] Although amyloid proteins in their native state can have both globular and disordered structures, through the aggregation into amyloid fibrils, transformation into cross β -sheet structures occurs.[3] However, existing evidence implies that S100A9 has a distinct aggregation pathway as S100A9 fibrils possess a significant amount of a-helical structures alongside β-sheets.[4] Here we report experiments that were conducted to elucidate aggregation tendencies of S100A8/A9 proteins and their amyloidogenic aggregates structures.

Questions addressed: S100A9 protein aggregation has been characterized previously,[4] but there are no detailed studies of S100A8 *in vitro* aggregation. Additionally, S100A9 distinct aggregation aspects remain unclear, including structural propensities of S100A9 amyloid fibrils. Therefore, in this study, we were investigating S100A8 and S100A9 fibril's secondary structures and morphologies, as well as their aggregation rate dependence on protein concentration and temperature.

Methods: Fourier-Transform Infrared (FTIR) Spectroscopy was conducted using Bruker Invenio S FTIR spectrometer. FTIR data was processed using GRAMS software; Circular Dichroism (CD) spectra were recorded using J-815 CD spectrometer (JASCO, Japan) and processed using BeStSel;[5] Atomic Force Microscopy (AFM) was performed using a Dimension Icon atomic force microscope (Bruker, Billerica, MA, USA). AFM images were adjusted using Gwyddion software;[6] Thioflavin T (ThT) Fluorescence Assays were monitored in a ClarioStar Plus (BMG Labtech, Ortenberg, Germany) plate reader and data processing was done using Origin software (OriginLab Corp, Northampton, MA, USA).

Results and discussion: FTIR and CD measurements revealed that S100A8 fibrils contain atypical a-helical motives, similarly to S100A9. a-helical aggregates structure may comply with S100A8 and S100A9 fibrils morphology, as AFM imaging indicates these aggregates as curvy (worm-like) fibrils, as well as spherical oligomers. To evaluate S100A8 and S100A9 aggregation propensities, ThT Fluorescence Assays were conducted under 37 °C and 50 °C temperatures. Aggregation kinetics results indicated that both proteins have a short lag-phase period and half-time values (t_{50}) of the amyloid formation kinetics elevate significantly in both concentration and temperature-dependent manner. In summary, our conducted research provides significant and novel insights into S100A8 and S100A9 aggregation, which is tightly connected to various pathologies.

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Superoxide Dismutase-1 Alter the Rate of Prion Protein Aggregation and Resulting Fibril Conformation

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Background: Aggregation of proteins/peptides in the form of amyloid fibrils is associated with the occurrence of various amyloidoses, including neurodegenerative disorders such as Alzheimer's disease, amyotrophic lateral sclerosis or prionopathies. Despite many years of research dedicated to understanding the onset and development of these diseases, only a few drugs and treatments have been discovered. In recent studies, it has been observed that the formation of amyloid fibrils can be influenced by the cross-interaction of two different proteins.[1] One example of such an interaction was reported by Akhtar et al, who observed that a deficiency in SOD1 accelerated the aggregation of mouse prion protein *in vivo*.[2]

Questions addressed: Since the previously mentioned report described the influence of SOD1 on the aggregation of prion proteins in vivo, it prompted a need for a deeper investigation into the matter. In this work, we analyzed how different concentrations of SOD1 influenced both the kinetic parameters of prion proteins aggregation, as well as the structure of the formed fibrils.

Methods: Mouse prion protein fragment 89-230 and different concentrations of SOD1 were dissolved in the reaction buffer (2 M guanidinium hydrochloride, 1x PBS, pH 7.4) and incubated at 37ºC with constant 600 RPM agitation. These conditions promoted the fibril formation of prion proteins and prevented the aggregation of SOD1.This allowed the possibility of only analyzing the aggregation reaction of prion proteins. The formed fibrils were then analyzed using Fourier-transform infrared (FTIR) spectroscopy and atomic force microscopy (AFM).

Results and discussion: It was discovered that the presence of SOD-1 increased the lag time of PrP fibril formation and reduced their apparent rate of replication. In addition to all this, a significant decrease in the fluorescence intensity of thioflavin -T was observed at the highest concentration of SOD-1. Analyzing the FTIR spectra, it was observed that SOD-1 affects not only the kinetics of aggregation, but also the secondary structure of the formed amyloid fibrils. The results of this experiment showed that SOD-1 promotes the stabilization of a certain secondary structure of the fibrils. In addition, AFM images showed that these fibrils also have distinct morphological features.

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Fold of the tau amyloid in PHF as defined by the monoclonal antibody MN423

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Background: Tau protein is an intrinsically disordered protein, which plays an important role in regulation of microtubules.[1] However, in the course of Alzheimer's disease (AD) tau protein detaches from the microtubule surface and forms highly compact straight filaments and paired helical filaments (PHF) with cross beta structure of the amyloid spine.[2,3] The fold of AD tau filaments is imprinted into conformational antibody MN423.[4]

Questions addressed: Several structures of the AD tau filaments have been recently elucidated using cryo-electron microscopy, however, certain parts of structure with low densities still remain unrevealed at both N and C terminal regions of the filament core.[3]

Methods: We are using MN423 and other specific monoclonal antibodies to induce pathological tau fold into recombinant tau protein by co-crystallization. Pure and homogeneous recombinant antibody Fabs were produced in ExpiCHO-S™ and FreeStyle™ CHO-S cells. Co-crystallization experiments were performed using both hanging and sitting drop techniques. Diffraction data were collected at synchrotron sources (PSI beamline PXI, EMBL DESY beamline PX13).

Results and discussion: We have used a new method for the antibody production in CHO cell line for the crystallization experiments. After crystallization screening for complexes of different antibodies with recombinant tau dGAE (297-391) we obtained crystals of the putative complexes of tau with AD fold. Afterwards we collected diffraction data of the chosen crystals. Crystals gave diffraction up to 1.5–3 Å. In the next steps we will process the data using XDS package, solve the phase problem by molecular replacement and refine the structure, which will likely contain structural details not revealed by available cryo-EM structures of tau filaments.

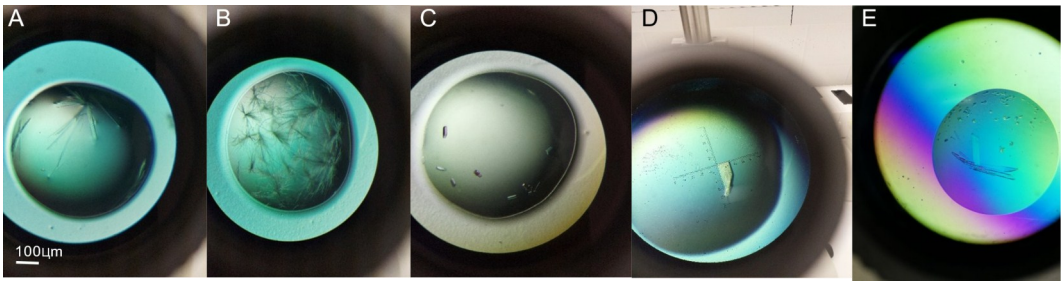

Figure 1. Crystals of different complexes: MN423Fab and tau (A, B); MN423Fab, DC11Fab and tau (C, D); DC25Fab, MN423Fab and tau (E).

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Sequence-based identification of amyloidogenic β-hairpins reveals a prostatic acid phosphatase fragment promoting semen amyloid formation

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Background: Amyloid fibrils rich in β-structure are a key feature of several diseases, the most prominent being Alzheimer's disease, Parkinson's disease and Diabetes Type 2. In their non-aggregated, intrinsically disordered monomeric state, amyloidogenic proteins can locally adopt hairpin conformations, which affects their aggregation. These hairpin conformations can be targeted by engineered proteins called β-wrapins, previous targets were Aβ ($ZA\beta_3$), αSyn (AS69) and IAPP (HI18). Here, potential β-hairpin-forming amyloidogenic segments in the human proteome were predicted with a bioinfomatics approach based on sequence similarity with the previously determined β-hairpins of Aβ, αSyn and IAPP.

Questions addressed: The questions addressed in this study were, is it possible to identifiy amyloidogenic β-hairpin peptides by a bioinformatics approach (Figure 1), and characterize the obtained hits experimentally.

Methods: Out of the large number of hits, a test set of eight protein segments were characterized in peptide format with respect to aggregation propensity and potential to adopt β-hairpin conformation, employing, e.g., NMR spectroscopy, CD, isothermal titration calorimetry, AFM and Thioflavin T assays.

Results and discussion: The majority of the investigated peptides formed Thioflavin T-positive aggregates upon incubation. NMR spectroscopy demonstrated that four of the eight tested peptides adopted β-hairpin conformation upon interaction with β-wrapin AS10. Structure determination of the complexes of the protein segments with AS10, performed by NMR using fusion constructs of the binding partners, revealed that they formed β-hairpin motifs highly similar to those previously observed for Aβ, αSyn and IAPP. Further analysis of one of the peptides, the prostatic acid phosphatase (PAP) fragment PAP(185-208) revealed a cross-talk and triggered aggregation of PAP(248-286), which is involved in functional amyloid formation in semen. In conclusion, a large number of potential β-hairpin-forming amyloidogenic protein segments could be identified in the human proteome with potential roles in amyloid formation.

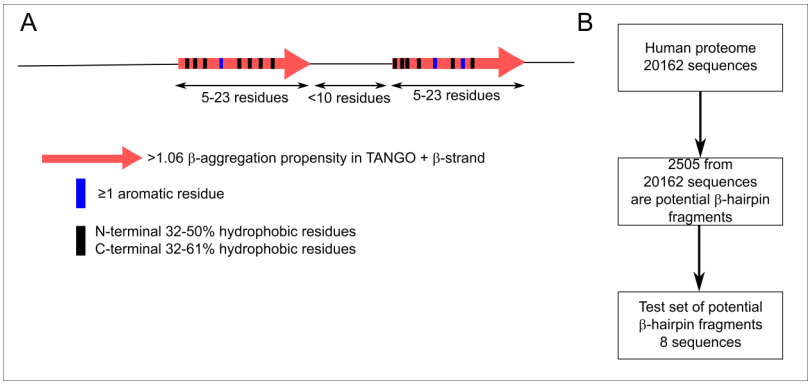

Figure 1. (A) The requirements for the β-hairpins to have based on the known β-hairpins from Aβ, α-synuclein and IAPP. (B) Overview of the bioinformatics results on the number of sequences.

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Targeting α-synuclein amyloid aggregates with the BRICHOS domain – basis for treatment of Parkinson's disease

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Background: Parkinson's disease (PD) is the second most common form of neurodegenerative disorder. Its pathogenesis is linked to the aggregation of α-synuclein protein (α-Syn) leading to accumulation of toxic oligomers and amyloid fibrils, which are found in patients' brains.[1]

Questions addressed: Identifying and controlling the pathways of aggregation have been challenging yet is the key for a detailed understanding of the molecular mechanisms of protein aggregation in neurodegenerative diseases. Due to repetitive failure of treatment in the past, new disease-modifying treatment strategies are currently highly desired. Naturally occurring chaperones, such as the BRICHOS protein domain, is part of the new class of endogenous inhibitors of amyloid toxicity and holds great promise for development of new therapeutic tools.[2]

Methods: The aggregation of α-Syn (WT and A53T) was analysed by Thioflavin T (ThT) fluorescence assay. α-Syn fibrils from PD mouse models were extracted using a sarkosyl-based extraction and the presence of α-Syn protein was analysed by dot blot. The seeding activity of extracted α-Syn fibrils were analysed by ThT assay.

Results and discussion: Here, we present *in vitro* α-Syn (WT and A30P mutant) aggregation inhibition mechanisms by BRICHOS, where BRICHOS efficiently prevents aggregation of WT (as shown in Figure 1) and A30P α-Syn in a concentration-dependent manner. Moreover, we demonstrate that α-Syn fibrils can be extracted from different mouse models and these fibrils are potent to seed recombinant α-Syn aggregation kinetics. These results provide a greater understanding of the molecular mechanisms of α-synuclein aggregation and the preventing effect of BRICHOS which could potentially be implemented in novel treatment approaches.

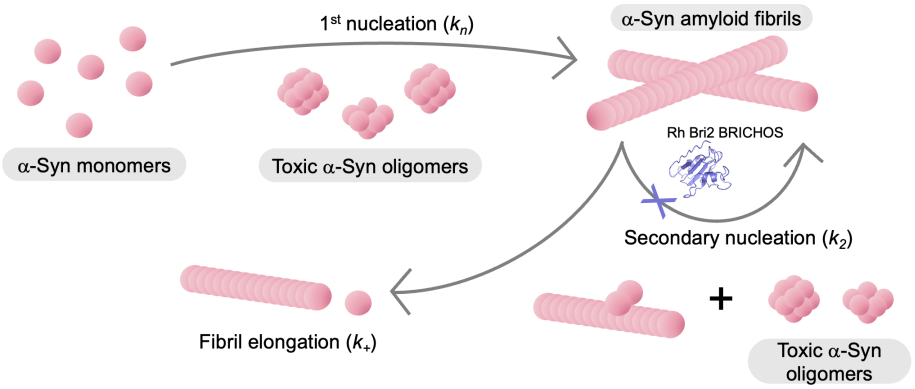

Figure 1. α-Syn protein aggregation mechanism and proposed rh Bri2 BRICHOS inhibition mechanism.

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Pro-inflammatory S100A9 Protein Affects Tau Protein Aggregation

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Background: Neurodegenerative diseases are among the most common disorders in the world. Unfortunately, despite intensive research, the understanding of the mechanism of these diseases is limited, and almost all existing treatments are symptomatic. Alzheimer's disease has attracted the most attention from scientists because it is the most common neurodegenerative disease, affecting about 50 million people worldwide. In addition to amyloid plaques composed of amyloid-β peptides, neurofibrillary tangles formed from the protein Tau are a hallmark of this disease and other tauopathies.[1] Amyloid-β aggregates (and α-synuclein aggregates in Parkinson's disease) have been shown to promote Tau aggregation. It has also been observed that the aggregation of these two peptides involves the pro-inflammatory protein S100A9, whose elevated levels in the brain are recorded after various head injuries.[2] It was found that in *post mortem* Alzheimer's patient brains Tau tangles colocalizes with S100A9 protein aggregates, raising the question about S100A9 involvement in this disease progression.

Questions addressed: There has been some speculation from the scientific community that neuroinflammation could induce Tau pathology; thus, it is feasible that S100A9 as a pro-inflammatory protein could be a culprit behind it or at least in part responsible. However, it is strange that there is not much information available, or studies performed to confirm or rule out the potential of the S100A9 protein or its aggregates to participate directly in Tau aggregation. And since amyloid aggregation mechanisms are quite complex and various links between proteins are still unknown, in this work we tried to elucidate the link between Tau and S100A9 proteins.

Methods: To determine the optimal aggregation conditions for Tau protein prior to experiments with S100A9 protein, polyanion heparin was used as an initiator of amyloid protein aggregation *in vitro*. Aggregation kinetics were followed using the amyloidophilic dye thioflavin T fluorescence (ThT) assay. Atomic force microscopy was performed to analyze the morphology of the formed aggregates. Fourier-transform infrared spectroscopy was used for the analysis of protein secondary structure..

Results and discussion: We examined the ability of the S100A9 protein and its aggregates to promote Tau aggregation. We observed that Tau aggregation is dependent on S100A9 aggregate formation as S100A9 monomers alone do not induce Tau aggregation, while S100A9 aggregates induce notable ThT fluorescence changes in the reaction mixture with Tau protein. Various ionic strength, pH, reducing environment conditions for S100A9 protein aggregation and co-aggregation with Tau protein were examined in the study. Also, we noted that fluorescence intensity change is considerably higher in mixtures of S100A9 with Tau than in S100A9 alone, indicating additional aggregate formation as ThT binds to aggregate structures. However, although kinetic data indicates amyloid-like aggregation pattern, after FTIR analysis we did not observe additional β-sheet formation in the presence of Tau. AFM data also did not confirm amyloid fibril formation, resulting aggregates appeared clump-like, indicating rather amorphous aggregation. Since S100A9 protein at the physiological conditions is charged negatively, while Tau is net positive, we hypothesize that the interaction is electrostatic in nature. This was confirmed using higher ionic strength aggregation condition where higher the NaCl concentration lowered fluorescence intensity change and aggregate formation. Thus, currently we theorize that Tau monomers are only electrostatically absorbed to S100A9 fibrils, rather than form amyloid-like fibrils.

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Using Lysozyme Amyloid Fibrils as a Means of Scavenging Aggregation-Inhibiting Compounds

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Background: Amyloid aggregation in the form of fibrils is linked with multiple amyloidoses, including neurodegenerative Alzheimer's and Parkinson's diseases.[1] The number of affected individuals is projected to further increase in the upcoming years and very few treatment modalities are available. Despite a large number of tested compounds, very few potential drug molecules manage to complete all stages of clinical trials and only a handful have been approved as viable treatments of amyloid diseases.[2] This makes it necessary to find new and better ways of identifying potential aggregation-inhibiting compounds.

Questions addressed: In this work, we examined whether there is a link between molecule anti-amyloid activity and their affinity towards such protein aggregates. In addition, we attempted to separate the fibril-bound compounds using three different approaches and analyzed their effectiveness at inhibiting the process of amyloid aggregation.

Methods: Lysozyme amyloid fibrils were prepared by dissolving hen egg-white lysozyme in PBS, containing 2 M of guanidinium hydrochloride, and incubating the samples at 60°C with constant agitation. Four different complexity inhibitor solutions were prepared from gallic acid, epigallocatechin-3-gallate and green tea leaves. The solutions were then combined with lysozyme amyloid fibrils, after which the fibrils were pelleted and washed multiple times with the buffer solution. The aggregates were then destabilized using either DMSO or guanidinium thiocyanate. The monomeric lysozyme was then removed from solution by using 10 kDa concentrators. The resulting solutions and the initial supernatants were examined using HPLC and insulin aggregation assay.

Results and discussion: When the inhibitor solutions were combined with lysozyme amyloid fibrils and then separated by centrifugation, the resulting supernatants did not possess any aggregation-inhibiting effect. This suggests that everything that could inhibit amyloid fibril formation became bound to the lysozyme fibrils. All three methods used to separate the fibril-bound molecules (diffusion, denaturation with guanidinium thiocyanate and destabilization with dimethyl sulfoxide) yielded solutions which were capable of slowing down insulin aggregation. Analysis of both the initial supernatants and the separated compound solutions revealed that there were multiple molecules, which could bind to lysozyme fibrils in each of the four different inhibitor solutions. These results show that it is possible to selectively separate anti-amyloid compounds from complex inhibitor mixtures, by exploiting their affinity towards amyloid fibrils.

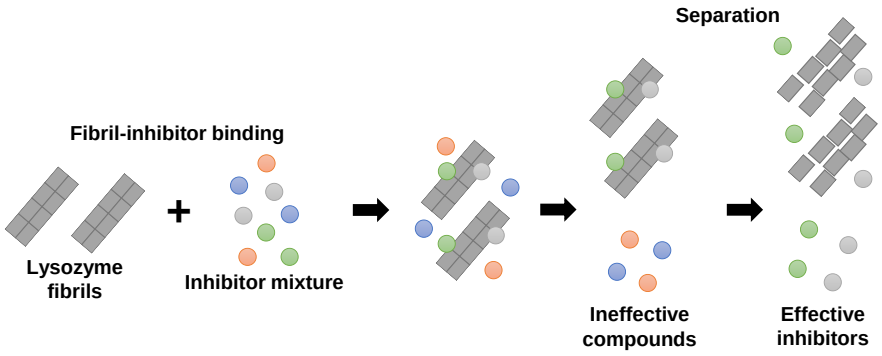

Figure 1. Process of effective anti-amyloid compound separation by using lysozyme amyloid fibrils.

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The impact of reading frequency upon fibrillation kinetics

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Background: It's long been known that agitation speeds up amyloid fibrillation kinetics,[1] a more recent discovery is that even the frequency with which a plate is read in a plate reader can have a major impact on fibrillation kinetics.[2] Not only is this an important aspect for reproducibility, but it could also reveal further insights into the molecular mechanisms of amyloid formation.

Questions addressed:
1. How does reading frequency affect the fibrillation kinetics of different amyloid systems?
2. What can this tell us about the molecular mechanisms involved?

Methods: This effect has mainly been investigated by ThT fluorescence in several Fluostar plate readers (BMG), using fresh & ultra-pure peptides (e.g. Aβ, α-synuclein & a fragment of tau). Reading frequencies have varied between continuously and every 30th minute.

Results and discussion: We see a clear effect for Aβ, α-synuclein and a tau fragment, which together with the reported effect on IAPP strongly indicates that this is a general phenomenon. For Aβ at 2.5 μM, preliminary results suggest that the effect can be as large as a 3-fold decrease in $t_{1/2}$, upon increased agitation from more frequent readings, as shown in Figure 1. We are currently investigating the underlying molecular mechanisms behind these observations. One possible explanation is that the increased movement of the plate resulting from the higher reading frequency could facilitate the detachment of secondary nuclei, a phenomenon well-known in crystallization.[3] Another possibility is that the fragmentation rate and/or primary nucleation are faster under mild agitation. This will be discussed further in the poster.

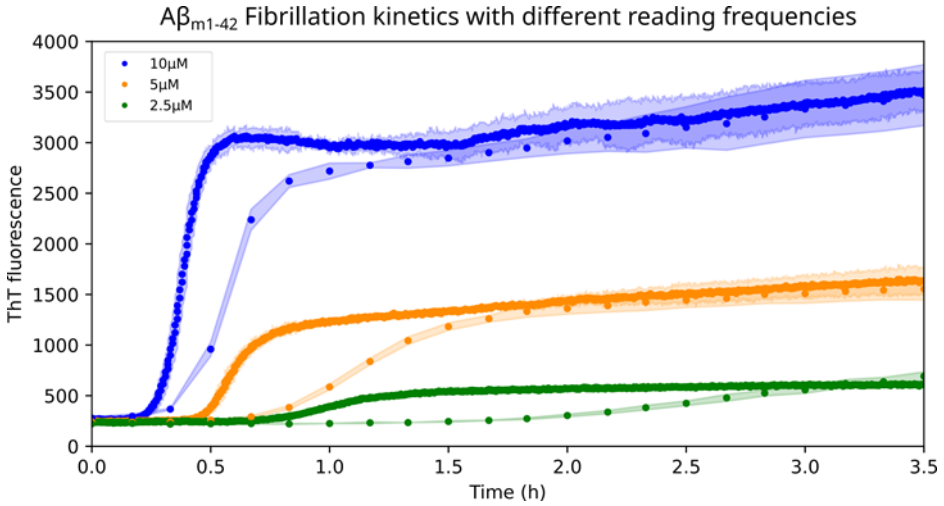

Figure 1. Fibrillation kinetics of Aβm1-42 at 3 different concentrations (10, 5 & 2.5 μM). Read either continuously (fast) or every 10 minutes (slow). Both fast & slow were done in the same plate reader (consecutive days), with protein freshly purified both times in the same system, from the same batch. Points are mean and shaded area is standard deviation, N=3.

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Cations' influence on amyloid aggregation of α-lactalbumin

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Background: α-lactalbumin (α-LA) is a relatively small, globular, calcium-binding protein found in the whey fraction of milk in all mammals and is well known for its molten globular state induced by Ca^{2+}-depletion. Despite that, there is limited knowledge regarding α-LA amyloid aggregation and its mechanism.

Questions addressed: Our study's goal was to examine the formation of α-LA amyloid fibrils (α-LAF) in the presence of cations (Mg^{2+}, Ca^{2+}, Na^{+}, K^{+}, NH^{4+} and Cs^{+}) in the form of chloride salts to distinguish differences in terms of kinetics, morphology and secondary/tertiary structure, aiming at their possible utilization as future bionanomaterials.

Methods: The kinetics of α-LA's amyloid fibrillization was followed by thioflavin T fluorescence assay. The infrared spectroscopy and near-UV circular dichroism were used to monitor and explain changes in the secondary structure of the native and fibrillar α-LA. The morphology of prepared amyloid aggregates was visualized by atomic force microscopy.

Results and discussion: We have shown that studied cations affect the conformation of native α-LA, the kinetics of its amyloid formation (Fig. 1), secondary structure and morphology of α-LAF in a different manner at studied salt concenterations (100 mM and 300 mM). Both salt concentrations stabilized α-LA's secondary structure, however the higher salts concentration significantly accelerated the aggregation process. The presence of divalent cations resulted in shorter fibrils with less β-sheet content. Moreover, strongly hydrated Mg^{2+} significantly altered α-LA's tertiary structure, followed by Na^{+}, $NH^{-}_{4}{}^{+}$, K^{+}, and weakly hydrated Cs^{+}. On the other hand, Ca^{2+}, despite being also strongly hydrated, stabilized the tertiary structure, supposedly due to its high affinity towards α-LA. Even so, Ca^{2+} was not able to effectively inhibit α-LA amyloid aggregation.

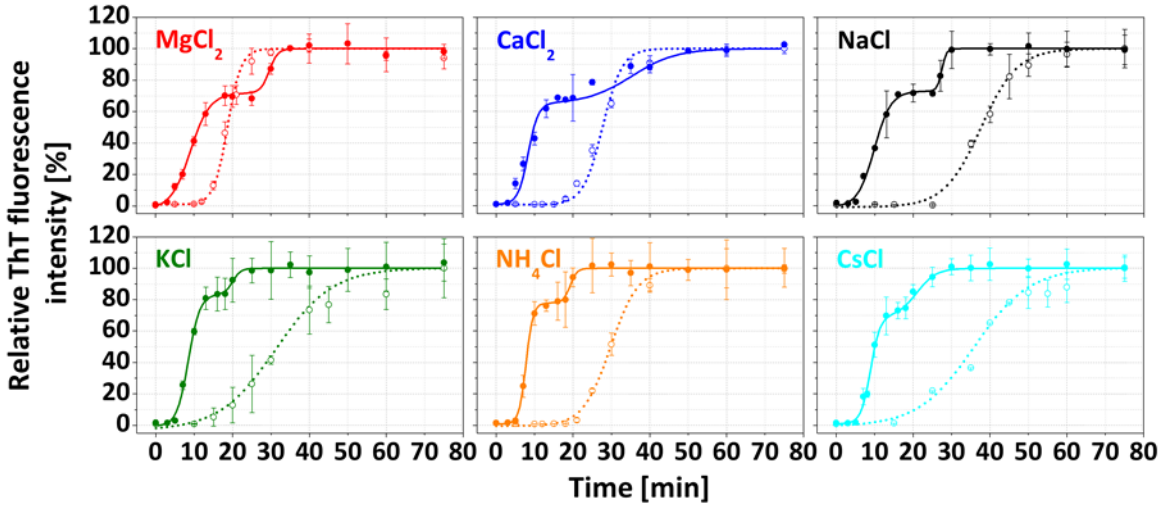

Figure 1. Effect of different salts on amyloid aggregation kinetics of α-LA: $MgCl_2$ (red), $CaCl_2$ (blue), NaCl (black), KCl (green), NH_4Cl (orange) and CsCl (cyan) at 100 mM (dotted lines, empty symbols) and 300 mM (solid lines, full symbols) concentrations. Error bars represent the average deviation of three separate measurements

Acknowledgements
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Met/Val129 polymorphism of the full-length human prion protein dictates distinct pathways of amyloid formation

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[+] Authors contributed equally

Background: Methionine/valine polymorphism at position 129 of the human prion protein, huPrP, is tightly associated with the pathogenic phenotype, disease progress, and age of onset of neurodegenerative diseases such as Creutzfeldt-Jakob disease or Fatal Familial Insomnia.

Questions addressed: This raises the question of whether and how the amino acid type at position 129 influences the structural properties of huPrP, affecting its folding, stability, and amyloid formation behavior.

Methods: Analyses of the 129M and 129V variants of recombinant full-length huPrP(23-230) by amyloid formation kinetics, circular dichroism spectroscopy, molecular dynamics simulations, and sedimentation velocity analysis revealed differences between both variants and the importance of the unstructured N- terminal half.

Results and discussion: Detailed biophysical characterization of the 129M and 129V variant of recombinant full-length huPrP (23-230) reveals differences in their aggregation propensity and oligomer content, leading to deviating pathways for the conversion into amyloid at acidic pH (Figure1). The 129M variant exhibits less secondary structure content before amyloid formation and higher resistance to thermal denaturation compared to the 129V variant, whereas the amyloid conformation of both variants shows similar thermal stability. Molecular dynamics simulations and rigidity analyses at the atomistic level identify intramolecular interactions responsible for the enhanced monomer stability of the 129M variant, involving more frequent minimum distances between E196 and R156, forming a salt bridge. Removal of the N-terminal half of the 129M full-length variant diminishes its differences compared to the 129V full-length variant and highlights the relevance of the flexible N-terminus in huPrP.[1]

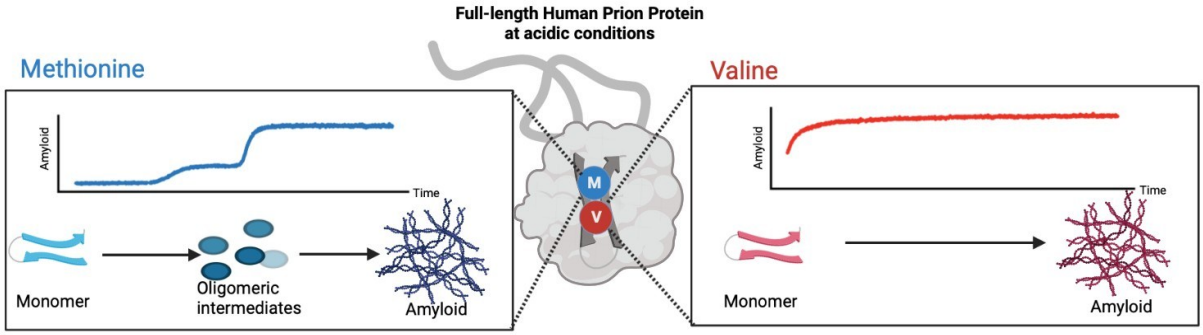

Figure 1. Differences in amyloid formation behavior of full-length huPrP 129M and 129V variant at pH 2 with further destabilization using GdnHCl. 129M variant forms oligomeric intermediates with limited ThT binding capacity, which undergoes structural rearrangement. 129V variant shows immediate amyloid formation.

References:

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Targeting toxic amyloid aggregates with the BRICHOS protein – basis for treatment of Alzheimer's disease

Rakesh Kumar, Laurène Adam, Shaffi Manchandra, Tanguy Le Marchand, Mirjam Brackha, Guido Pintacuda, Jens Pahnke, Per Nilsson Per Nilsson, Jan Johansson, Axel Abelein*

Alzheimer's disease and Parkinson's disease are the two most common forms of neurodegenerative disorder. Their pathogeneses are linked to strikingly similar molecular mechanisms where amyloid deposition of the amyloid-β peptide (Aβ) and α-synuclein protein are found in patients' brains, respectively. While most recent insights have been obtained *in vitro*, knowledge on how these findings translate to the *in vivo* situation in the brain is currently highly desired. Moreover, due to the drastic failure rate of treatments in the past, new effective therapeutics are necessary to target generation of toxic species. Here, naturally occurring anti-amyloid proteins, such as the BRICHOS protein domain, have the potential to be implemented in novel treatment approaches. Here, we present structural insights and neurotoxicity of *in vivo*-derived fibrils from different AD mouse models, and how BRICHOS modulates them. These results provide a greater understanding of the molecular mechanisms of Aβ aggregation that may occur *in vivo*.

Fibrillation of peptides from soy protein isolate

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Background: Industrial side-stream products such as soy protein isolates (SPI) have been used to produce non-pathological amyloid-like protein nanofibrils (PNFs), a biobased and sustainable alternative to material-building-block from petroleum-based polymers. It is known that these amyloid fibrils are formed through acid-hydrolysis-based reactions, where peptide-building blocks are released and aggregated at high temperatures and low pH. Indeed, SPI forms PNFs of mainly curly morphology, but their peptide-building blocks form straight PNFs when fibrillated individually. In that light, we study the aggregation of a peptide segment (BB2) from the beta-chain of soy beta-conglycinin, one of the peptide- building blocks of SPI-PNFs together with other soy glycinin derived peptides. We proceed to compare the BB2 kinetics with SPI rate of hydrolysis at different temperatures to understand how BB2 aggregation correlates with the one of raw SPI.

Questions addressed: (1) what thermodynamic processes determine the morphology switch in SPI- PNFs and how does BB2 participate in that process, (2) how SPI hydrolysis at different temperatures correlates with BB2 fibrillation, (3) how morphologically different are SPI-PNFs and BB2-derived PNFs.

Methods: Atomic force microscopy (AFM), UV/vis and fluorescence spectroscopy, circular dichroism (CD) spectroscopy, SDS-page, size-exclusion chromatography (SEC).

Results and discussion: Aggregation kinetics of BB2 at three different temperatures (25, 35 and 45 °C) and various concentrations suggest that BB2 fibrillation is a secondary nucleation-driven process. Fibrillation proceeds even at room temperature, which is equivalent to many amyloid-like proteinaceous systems like Aβ. Additionally, nano-morphometric statistical analysis using atomic force microscopy (AFM) shows no considerable changes in the morphology or physical properties of BB2 fibrils in the investigated temperature interval (Figure 1).

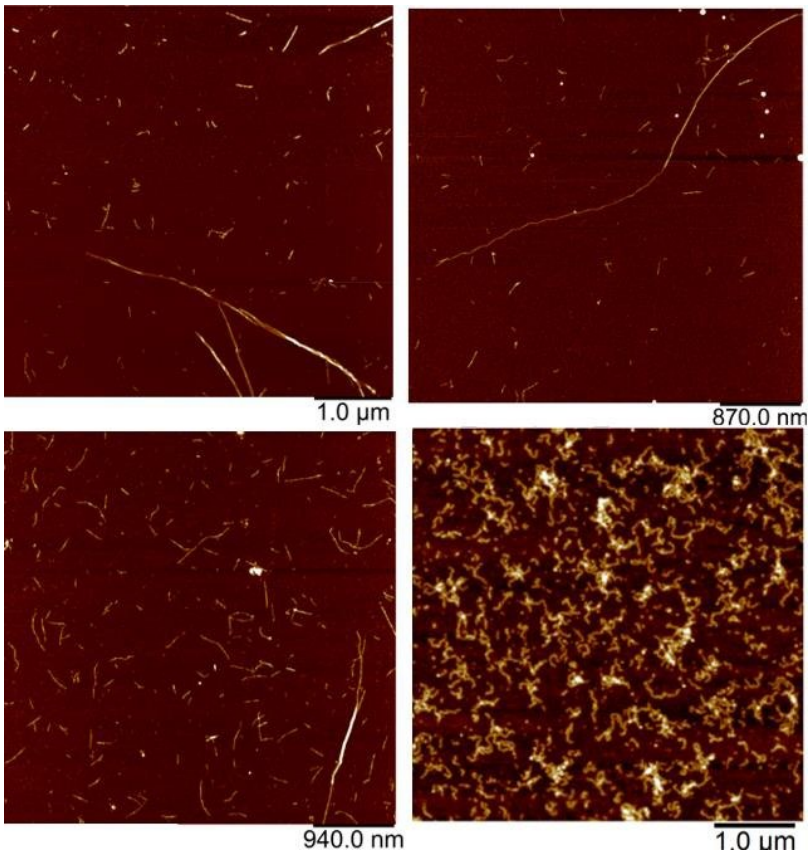

Figure 1. AFM images of BB2 fibrils incubated at 25 °C (top left), 35 °C (top right), 45 °C (bottom left) and SPI (bottom right).

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Aggregation of TMEM106B protein and its possible interactions with Tau

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Background: Aggregation of amyloid proteins into fibrillar structures is associated with several dozen amyloidoses, including neurodegenerative diseases, which are among the most common disorders in the world, the most common of which – Alzheimer's disease – affects about 50 million people. It is predicted that these numbers will more than triple in the next 30 years. Unfortunately, there is no ultimate cure for it, like for any other tauopathy, so the existing treatment is only symptomatic.
There are two hallmarks of Alzheimer's disease: amyloid-β plaques and Tau protein tangles. The latter are the engine of disease progression, but are characteristic not only of Alzheimer's pathology, but also of other tauopathies.[1] Recently, it was discovered that in some diseases together with Tau tangles amyloid structures of a lysosomal protein, TMEM106B, can be found. No *in vitro* studies of TMEM106B interactions with Tau protein have been reported so far, and the main knowledge about the aggregation of this protein comes from postmortem brain research.[2] It is hypothesized that the aggregation of the TMEM106B proteolysis product can directly stimulate Tau protein aggregation and thus activate the progression of various neurodegenerative diseases – tauopathies.[3]

Questions addressed: Since complex amyloid protein aggregation mechanisms are involved in tauopathies development, it is crucial to elucidate all ways these disorders form, helping to find new possible drug targets. Therefore, here we aim to answer a question how TMEM106B aggregates *in vitro* and does it affect Tau aggregation.

Methods: Tau2N4R and TMEM106B genes were fused at N-termini with ULP1 protease cleavable His-Sumo tag. Tau2N4R gene was inserted into pET Champion His-Sumo vector using TA cloning method, while His-SUMO-TMEM106B gene was inserted into pET28A expression vector by restriction cloning. Both proteins were purified using Ni^{2+} ion affinity chromatography method. After His-Sumo tag cleavage both proteins went through size exclusion chromatography. Aggregation kinetics are followed by thioflavin T fluorescence assay. Fibril morphology will be observed using atomic force microscopy. Fourier-transform infrared spectroscopy will be used for secondary structure analysis.

Results and discussion: In this work we have purified TMEM106B specific fragment (120-254 aa), which was reported to form amyloid structures *in vivo*. Within next months we are going to study amyloid fibril formation *in vitro*, analyze secondary structure and morphology of formed aggregates. In addition, we would like to test possible effect of TMEM106B on Tau aggregation and analyze resulting structures. The obtained data will be presented in ADAM conference in November.

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Exploring Effect of Flavones on Amyloid Aggregation of Mouse Prion Protein and Aβ42

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Background: Protein aggregation into amyloid fibrils is linked to multiple neurodegenerative disorders, such as Alzheimer's, Parkinson's or Creutzfeldt-Jakob disease.[1] However, the formation of amyloid fibrils not only arise from the aggregation of a specific protein, but the aggregation process between proteins of different nature can be influenced by their interactions.[2] For example, co-aggregation between disease-associated protein amyloid-β and cellular prion protein has a key role in Alzheimer's disease. Amyloid inhibitors that could overset such co-aggregation or even reduce toxicity can be considered as potential anti-amyloid drugs.

Questions addressed: The coaggregation process of cellular prion protein and Aβ is slower compared with an Aβ alone, the resulting aggregates can be cytotoxic. Natural inhibitors such as flavones can act to affect amyloid fibril formation processes or reduce cellular toxicity.

Methods: The aggregation of mouse prion protein mutant and Aβ alone as well as the influence of flavones to aggregation kinetics was revealed by Thioflavin T (ThT) fluorescence assay. To determine the interaction between Aβ and MoPrP mutant, the morphological variability was observed by using atomic force microscopy. The cell viability study was used to evaluate MoPrP 89-230, Aβ42 and flavones toxicity on human neuroblastoma cells (SH-SY5Y).

Results and discussion: ThT fluorescence assay showed MoPrP 89-230 inhibitory effect on amyloid-β, where morphology study indicated formation only of Aβ42 amyloid fibrils. Meanwhile, analysis of cell viability revealed that co-aggregates formed in the cellular environment were more toxic compared with final amyloid fibrils. Reduced cytotoxicity of pre-formed amyloid fibrils can be determined by structural stability or size. The study of flavones' influence on the amyloid co-aggregation showed that a small part of compounds inhibited the aggregation process of Aβ and MoPrP 89-230 as well as increased cell viability. These results can be addressed to flavone related stability of Aβ protein or conformational changes of protein oligomers.

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DC11 reactive tau conformation as preaggregation form of AD-associated protein tau

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Background: By hyperphosphorylation of amino acid residues of the tau polypeptide chain, or upon truncation, tau dissociates from microtubules. At the same time disintegration of microtubule and aggregation of tau monomers occur, leading to nerve cell damage. Although it has not yet been confirmed whether this process is the trigger for Alzheimer's disease (AD), the presence of insoluble aggregates of tau has been shown to be a hallmark of AD.[1]

Questions addressed: Neither the cause of Alzheimer´s disease, the mechanism of its development nor the treatment are still known. One of the many hypotheses favours the microtubule protein tau to be the main actor in the AD origin. Monoclonal antibodies appear to be a useful tool to obtain the transient tau structures, mainly serving as surrogate binding partners enabling crystallization of IDPs.[2,4] MAb DC11 discriminates very strictly between physiological tau proteins and truncated tau peptides, implying the presence of conformational epitope of tau.[2,3] Complexes of tau peptides with antibody Fab fragments can provide detailed molecular insights exploitable in AD drug and vaccine development.

Methods: $Tau_{321-391}$ was expressed in competent *E. coli* BL21-1 (DE3) cells and isolated.[5] DC11Fab was produced in CHO cells and purified using affinity chromatography with protein G. For crystallization of complexes, tau peptide was incubated with DC11Fab in 1.5:1 molar ratio. Crystallization was performed by the vapour-diffusion technique in sitting drops in an MRC 96-well crystallization plate. Synchrotron data was collected at P13 beamline operated by EBML Hamburg at the PETRA III storage ring (DESY, Hamburg, Germany). Diffraction data were processed using XDS.

Results and discussion: We focused on the crystallization of the Fab fragment of DC11 antibody with $tau_{321-391}$ to approximate the transition of tau from physiological to pathological conformation. Crystals of DC11Fab alone gave diffraction up to 1.4 Å, while crystals of DC11Fab complexed with $tau_{321-391}$ gave diffraction up to 1.74 Å as shown in Figure 1.

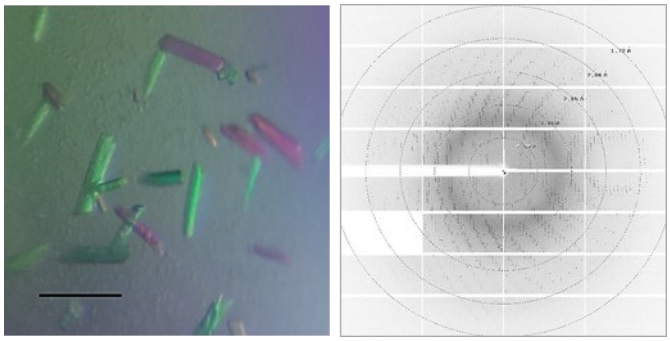

Figure 1. Crystals of complex DC11Fab/tau321-391 grown in 0.1 M PCTP, pH 5.0, 25 % w/v PEG 1500 and corresponding diffraction pattern.

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The Effect of dGAE Fragment Concentration on Its Aggregation on Tau Protein and Self-Aggregation

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Background: Protein aggregation into amyloid fibrils is associated with several widespread neurodegenerative disorders. Alzheimer's disease is one of the most prominent cases, with an ever-increasing number of afflicted patients. The disorder is characterized by memory loss and is considered to be caused by amyloid-β extracellular plaques and neurofibrillary tangles made from hyperphosphorylated Tau. The main physiological function of the Tau protein is to maintain microtubule dynamics and promote polymerization. *In vitro*, Tau protein filament formation is induced by the presence of polyanions such as heparin. A truncated form of Tau protein (dGAE), which can assemble into filaments without polyanions, forms a proteolytically stable core region in full□length Tau paired helical filament (PHF).[1] The dGAE fragment could serve as a model system for the search for inhibitors of Tau protein aggregation.[2]

Questions addressed: Considering the rise of Alzheimer's disease incidences worldwide, understanding the process of amyloid aggregation is a crucial step in developing drugs. Research of dementia is heavily focused on the process of how pathogenic Tau can spread throughout the brain, as it may provide a key therapeutic target for slowing the development of tauopathies. There are a lot of disputed questions concerning the physiological and pathological consequences of PHF-core Tau self-assembly, therefore we aim to elucidate the influence of dGAE protein on Tau protein aggregation.

Methods: Tau 2N4R gene was inserted into pET Champion His-SUMO vector by TA cloning method to create the ULP1-cleavable N-terminally His-SUMO-tagged Tau. The recombinant His-SUMO-tagged Tau (2N4R isoform) protein was purified by immobilized metal affinity chromatography and consequently cleaved by the ULP1 protease to remove His-SUMO -tag. A truncated form of Tau protein (297□391) (dGAE) was purified by cation exchange chromatography. Both proteins were purified further by size-exclusion chromatography. We used purified Tau 2N4R protein and dGAE fragment for monitoring the amyloid aggregation process. All performed aggregation kinetics were followed using a thioflavin-T fluorescence assay. Atomic force microscopy was performed to analyze the morphology of the formed aggregates.

Results and discussion: In this work, we examined how the presence of dGAE influenced the aggregation of Tau protein, using a range of different Tau/dGAE concentrations. We observed that when both proteins were present in solution together, the aggregation kinetic curves differed from the control samples both in end-point fluorescence intensity value distributions, as well as the actual shapes of the curves. Interestingly, there was a peculiar and unexpected dependence between the changes to aggregation kinetics and the Tau/dGAE ratios, with a reduced level of co-interaction detected at intermediate Tau protein concentrations.

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Ability of the BRICHOS domain to prevent neurotoxicity and fibril formation are dependent on a highly conserved Asp residue

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Background: Proteins and peptides can self-assemble into highly ordered fibrillar structures as well as into smaller oligomers, which are linked to severe human amyloid diseases. Molecular chaperones can prevent both these types of protein aggregation, but to what extent the respective mechanisms are overlapping is not fully understood. Amyloidogenic polypeptides can be expressed as proproteins which are subjected to proteolytic cleavage to release the amyloid forming fragments. Some of these proproteins contain the anti-amyloid chaperone domain BRICHOS. Mutations in the BRICHOS domain or in the proproteins are associated with different protein misfolding and amyloid diseases, but the underlying pathogenic mechanisms are largely unknown.

Questions addressed: We aimed to investigate the pathogenic mechanisms that regulate BRICHOS anti-amyloid chaperone activities.

Methods: We analyzed all so far deposited 3 355 BRICHOS containing proproteins by bioinformatics tools. Functions of BRICHOS domains were studied by biochemical and biophysical methods, kinetics analysis, immuno-electron microscopy, electrophysiological measurements on mouse brain slices.

Results and discussion: The capacity of the BRICHOS domain to inhibit amyloid-associated neurotoxicity and fibril formation, respectively, is oppositely affected by mutations of a phylogenetically conserved Asp, whereas the capacity to suppress non-fibrillar, amorphous protein aggregation is not affected. Moreover, conformational changes occur as a result of Asp to Asn mutations, and those changes can partly be mimicked by lowered pH and the conserved Asp titrates with an apparent pKa between 6 and 7. Interestingly, the Asp is evolutionarily highly conserved in >3000 analysed BRICHOS domains but is replaced by Asn in some BRICHOS families. The chaperoning capacities of BRICHOS domain against amyloid neurotoxicity and fibril formation can apparently be modulated by a conserved Asp in response to pH changes, suggesting the possibility that the microenvironment may affect BRICHOS function and providing insights on amyloid disease development.[1]

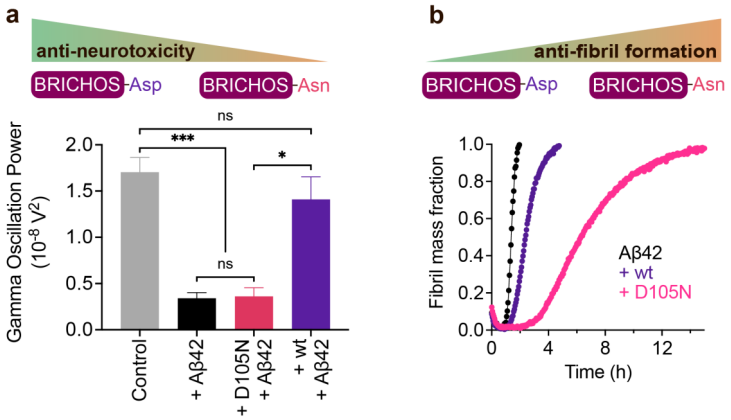

Figure 1. Effects of Asp to Asn mutation on BRICHOS capacities against amyloid-β (Aβ42) neurotoxicity and fibril formation, respectively. (a) Normalized γ-oscillation power of hippocampal slices from wildtype mice. (b) Activity comparison of wildtype BRICHOS and BRICHOS D105N against Aβ42.

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Bri23 binds to Bri2 BRICHOS domain and diminishes its anti-amyloid activity but not the canonical chaperone activity

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Background: Soluble peptides and proteins can convert into insoluble toxic amyloid aggregates, leading to more than 50 devastating amyloid diseases, including the severe neurodegenerative disorder of Alzheimer's disease (AD). The anti-amyloid chaperone domain, BRICHOS, is present in proteins containing a region with high propensity for amyloid formation and can be grouped into 13 otherwise unrelated protein families. The biological role of BRICHOS is apparently to protect amyloidogenic "client peptide" regions of their precursors from forming amyloid, and mutations in the BRICHOS domain can lead to human disease.

Questions addressed: The BRICHOS domain of dementia-relevant Bri2 protein, expressed in peripheral tissues and the brain, has been shown to prevent fibrillar amyloid formation and non-fibrillar amorphous aggregation of "non-client peptides, including AD-associated amyloid-β 42 (Aβ42) peptide;[1,2] however, the underlying molecular mechanism is still unclear.

Methods: The tertiary structure of human Bri2 BRICHOS-Bri23 (Bri23 is the C-terminal 23 aa region of Bri2 protein with high amyloid propensity) was predicted by AlphaFold. We recombinantly prepared human Bri2 BRICHOS-Bri23 protein, which was further characterized by thioflavin T (ThT) assay, transmission electron microscopy (TEM) and model reconstruction, and kinetics analysis. Further, interactions between Bri2 BRICHOS and the isolated Bri23 peptide were investigated by small-angle X-ray scattering (SAXS) and microscale thermophoresis (MST).

Results and discussion: The AlphaFold prediction showed that Bri2 BRICHOS-Bri23 adopted a globular structure, and Bri23 folded into a β-hairpin structure that folded into the BRICHOS hydrophobic core region between the central β-sheet and a-helix 1 (Fig. 1). The recombinant Bri2 BRICHOS-Bri23 formed monomers, dimers and high-molecular-weight oligomers that share similar overall 3D structure as Bri2 BRICHOS oligomers. ThT measurements showed that Bri2 BRICHOS-Bri23 is inactive in suppressing Aβ42 amyloid fibril formation, whereas, intriguingly, the canonical chaperone activity against non-fibrillar amorphous protein aggregation was not affected. Further, SAXS and MST measurements confirmed the individual Bri23 peptide can bind to recombinant Bri2 BRICHOS with micromolar affinity. These results indicate that the C-terminal Bri23 peptide can bind to the amyloid binding site of BRICHOS, which is decoupled from the binding site of the amorphous protein aggregation, giving general insights into how molecular chaperone interacts with different substrates.

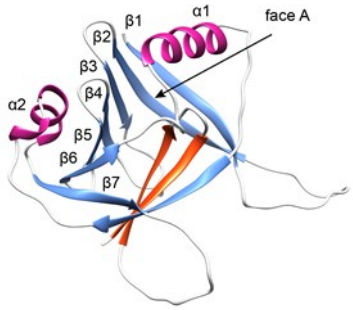

Figure 1. Predicted Bri2 BRICHOS-Bri23 tertiary structure by AlphaFold. The Bri23 is shown in orange, while BRICHOS domain is in blue with helixes in purple.

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Extracts from Chinese herbs with anti-amyloid and neuroprotective activities

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Background: A great effort is being devoted towards the search for amyloid fibrils inhibitors and/or compounds with high dissociation activity against amyloid fibrils of Aβ peptides, considering the critical link of amyloid fibrils with Alzheimer's disease. Moreover, understanding such compounds' interaction with Aβ fibrils at a molecular level is required to design novel therapeutic agents. Many Chinese herbs are well known for their neuroprotective and anti-oxidant properties.

Questions addressed: Extracts of *Salvia miltiorrhiza* and *Anemarrhenae asphodeloides*, tanshinone IIA (tanIIA), salvianolic acid B (Sal B) and sarsasapogenin (ML-1), were selected to study their anti-amyloid and neuroprotective properties. Moreover, derivatives of sarsasapogenin (ML-2, ML-3 and ML-4) have been prepared by the addition of modified carbamate moiety.

Methods: We have studied their ability to dissociate amyloid fibrils of $A\beta_{42}$ peptide using thioflavin T assay, circular dichroism (CD) spectroscopy and atomic force microscopy (AFM). The binding site and affinity of compounds to amyloid fibrils were determined using *in silico* methods. Moreover, we have tested the effect of compounds on the survival and cell cycle of SH-SY5Y cells, activity against NO- and H_2O_2-induced toxicity, and their ability to promote neuritic outgrowth.

Results and discussion: Sarsasapogenin derivative ML-4 containing carbamate moiety with N-heterocycle, tanshinone IIA and salvianolic acid B have a strong potential to dissociate $A\beta_{42}$ fibrils within low micromolar range. *In silico* data imply that active compounds bind to regions of $A\beta_{42}$ fibrils responsible for fibrils stabilization. Binding to the residues involved in the formation of the salt bridge may lead to destabilization and further disruption of fibrillar structure.
Evaluation of neuroprotective activities showed that sarsasapogenin and its derivatives were the most potent compounds. Derivatives ML-2, ML-3 and ML-4 possess the antioxidant properties with the ability to protect SH-SY5Y cells against H_2O_2-induced cytotoxicity. Moreover, derivatives ML-2 and ML-3 reduced NO production in LPS-induced RAW264.7 cells. Derivative ML-4 significantly promoted neuritic outgrowth compared to untreated SHSY5Y cells.
The reported data highlight the possibility of using active compounds to design novel treatment agents for Alzheimer's disease.

Acknowledgement:
This work was supported by the Slovak Research and Development Agency [APVV-18-0284; APVV SK-CN-2017-0033]; Slovak Grant Agency VEGA [02/0176/21 and 02/0030/18]; by the Operational Programme Integrated Infrastructure, the project "NANOVIR", ITMS: 313011AUW7, co-funded by ERDF; the China 111 Project (Grant BP0719034) and Exchange project of the 8th regular meeting of China Slovak intergovernmental cooperation, Chinese Ministry of Science and Technology ([2017] NO.21).

On the Self-Assembly of DNAJB6 Chaperone

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Background: The human chaperone DNAJB6b has the potential to increase solubility of several proteins involved in protein aggregation diseases, as well as suppress the primary nucleation of the amyloid formation.[1,2] Understanding how DNAJB6b operates on a molecular level is potentially key to design inhibitors against amyloid formation. The first step towards this understanding is to gain knowledge about how DNAJB6b behaves on its own.

Questions addressed: How does the self-assembly of DNAJB6b depends on the protein concentration?

Methods: Microfluidic diffusional sizing (MDS) has been used to examine the hydrodynamic radius of DNAJB6 assemblies at concentrations varying from 20 nM to 83 μM. Conditions: 20 mM sodium phosphate buffer, 0.2 mM EDTA, pH 8.0 at room temperature. Two different approaches of using the MDS technique was used. Either the DNAJB6b was non-labelled initially (Se Figure 1 (left) for detailed set-up) and post-labelled with OPA for detection of diffused portion. The other approach used pre-labelled DNAJB6b (attached Alexa Fluor™ 647), enabling a broader concentration range of detection. Triplicates or more have been made for all concentrations and labeling set-ups.

Results and discussion: The highly overlapping curves, seen in Figure 1 (right), of the two methods indicates that the pre-labelling did not alter the mean assembly sizes significantly. A critical aggregation concentration of around 100 nM can be seen, with free monomers (and/or dimers) at lower concentrations, corresponding to 2-3 nm in radius. Furthermore, a plateau of the mean size of assemblies is seen at concentrations higher than 10 μM. This size is around 12 nm in hydrodynamic radius.

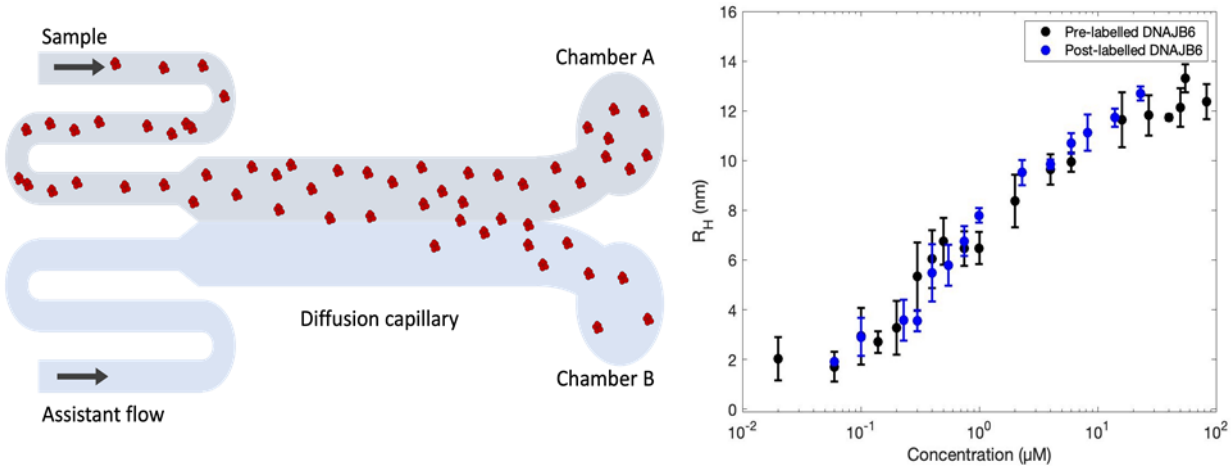

Figure 1. To the left: schematic of the MDS technique. Laminar flow of sample and assistant flow. Due to diffusion, some particles will end up in Chamber B. The portion of diffused particles reports on the mean hydrodynamic radius. To the right: DNAJB6b mean hydrodynamic radii at various protein concentrations (note the logarithmic scale), measured with MDS in both post- and pre-labeled set-ups.

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Solubility of tau

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Background: Determining the solubility of a molecule can seem trivial and easy, just add more solute and see how much can be dissolved before it precipitates. However, due to the high metastability of proteins and the intricate mechanisms of amyloid fibril formation the task is not necessarily easy. One way to determine the apparent solubility is to have a supersaturated solution of your amyloid peptide of interest, give the system enough time to reach equilibrium, then separate the insoluble fibrils from the soluble monomer and determine their concentration. This has been done in the lab with the Aβ40 peptide.[1] Tau on the other hand is notoriously soluble and does not aggregate in vitro without the addition of polyanionic inducers like heparin or RNA. In this work a smaller fragment of the full-length tau protein, spanning amino acids 304-380C322S has been used. Cryo-EM structures of ex-vivo tau tangles from Alzheimer disease brains, show that this fragment span the amyloidogenic core of tau[2] and this fragment has been shown to aggregate on its own in vitro without the need of inducers.[3]

Questions addressed: Understanding of how intrinsic factors (different isoforms, post translational modifications) and extrinsic factors (pH, salt concentrations, cellular components, chaperones) affect the monomeric solubility. Will help in the overall understanding of the molecular driving forces underlying the pathogenesis of amyloid diseases. This could in turn aid the development of effective therapeutics.

Methods: ^{15}N & ^{3}H isotope recombinant protein expression, tag free protein purification. Separation of monomers and fibril by filtration and ultracentrifugation. Concentration determination by liquid scintillation counting, primary amine reactive probes (OPA, Ninhydrin), HPLC, MALDI-MS and microfluidic diffusional sizing.

Results and discussion: Preliminary data has found the solubility of this fragment to be around 1.8 μM, determined using liquid scintillation counting (LSC), a method which would allow measurement in more complex sample matrices like cerebral spinal fluid (CSF). LSC have the potential to become an automated high-throughput method to screen how compound libraries affect the solubility of amyloids in *in vivo* mimicking systems like CSF.

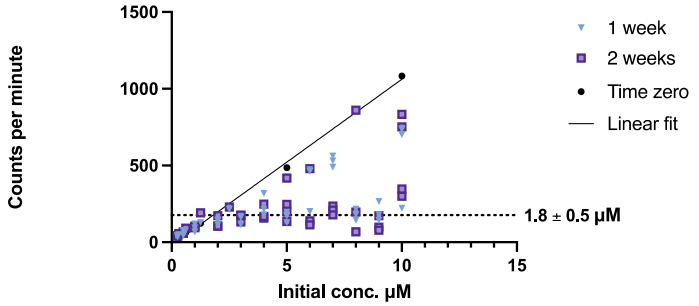

Figure 1. Apparent solubility of Tau 304-380C322S in 20 mM sodium phosphate pH 8, measured by liquid scintillation counting of ^{3}H labeled monomers at equilibrium, after separation from fibrils by filtration.

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Amyloid fibrils from AD mouse brain tissue for high resolution structural studies

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Background: Alzheimer's disease (AD) research focus has increased over the last years due to its high socio-economic impact on society and growing burden, affecting the quality of life of patients and their relatives. Different hypotheses have attempted to describe the origin of AD, being the amyloid hypothesis the most popular. This hypothesis suggests that AD pathogenesis is the result of amyloid-beta (Aβ) aggregation into insoluble fragments such as oligomers, fibrils and plaques after the cleavage of the amyloid precursor protein (APP) by β- and γ-secretase. AD animal models in combination with modern technologies can be useful tools for the study of the disease-associated structures such as Aβ fibrils.

Questions addressed: Different Aβ fibril structures exist, but only one *ex vivo* Aβ structure from an AD mouse model, the App^{NL-F} knock in mouse, has being determined at atomic resolution up to date.[1] It is believed that fibril structures might be disease specific, therefore, this study aims to describe the AD Aβ fibril structures of the APPswe/PSEN1dE9 mouse line and the cerebral amyloid angiopathy (CAA) TgSwDI mouse line containing the Swedish (APP KM670/671NL), Dutch (APP E693Q) and Iowa (APP D694N) mutations.

Methods: The APPswe/PSEN1dE9 and TgSwDI brain tissue from aged mice was immunohistochemically characterised. Aβ sarkosyl insoluble fibrils were purified based on a previously reported protocol.[1] that was slightly modified. The resulting fibrils were further analysed by negative staining EM, immunogold labelling and cryo-EM. Moreover, these structures were also compared with a well-established *in vitro* Aβ fibril structure.[2]

Results and discussion: Immunohistochemistry of the tissue showed a clear plaque pathology in both mouse models and the Aβ fibrils were successfully extracted from the brain with a concentration that is suitable for a cryo-EM structure reconstruction. Nonetheless, it is worth to highlight that the fibril extraction protocol was sensitive to changes in temperature, sarkosyl concentration and frequency of homogenization, therefore, the procedure was optimised accordingly.
Immunogold-staining EM identified the isolated fibrils as Aβ fibrils. Furthermore, preliminary cryo-EM data sets of the Aβ fibrils from both mouse lines showed a dimeric composition with a 2-fold symmetry, but revealed a different morphology compared to each other. This ongoing study intends to describe more detailed structural characteristics of APPswe/PSEN1dE9 and/or TgSwDI fibrils to a further extent taking into consideration that fibrils derived from transgenic mouse brain tissue can represent an infinite fibril source for future structure-function related research.

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Identification of lipid changes associated with Parkinson's Disease and alpha-synuclein pathology in dopaminergic-like neuronal cells

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Background: Parkinson's Disease (PD) is the second most common neurodegenerative disorder at older ages and is characterised by the loss of dopaminergic neuronal cells and the deposition of protein inclusions called Lewy Bodies that are mainly composed of the protein α-synuclein (αS) and other molecules such as lipids.[1–4] αS has been proposed to interact with lipid membranes as part of its functional role but this interaction was also found to initiate the formation of amyloid fibrils that resemble those found in the brain of patients.[5,6] Moreover, mutations in the *GBA* gene, that encodes the enzyme Glucocerebrosidase (GCase), is the most important risk factor for PD and can lead to the alteration of membrane composition and increased levels of aggregated αS in cell cultures and *in vivo*.[7,8]

Questions addressed: Here, we investigate how a decrease in GCase protein and activity levels may affect lipids and αS levels of differentiated SH-SY5Y cells, a cellular model of dopaminergic neuronal cells, and their organelles.

Methods: We induce a decrease in GCase activity and protein level in differentiated SH-SY5Y cells using conduritol β Epoxide (CBE) treatment and *GBA* knock down (KD), respectively. Lipids were extracted from the whole cells or from isolated organelles using a mixture of organic solvents and were then used either to prepare model membranes for biophysical experiments or to be analysed using shotgun lipidomics. We then studied how changes in lipid composition associated with changes in GCase protein and activity levels may affect αS aggregation using kinetic measurements of its aggregation in the presence of model membranes made with lipids isolated from the different SH-SY5Y cell preparations.

Results and discussion: We found that CBE treatment of differentiated SH-SY5Y cells led to a 99% decrease of GCase activity and a slight increase in the protein level whereas *GBA* KD led to a decrease of both the protein and activity levels by ca. 30%. Such a decrease in GCase activity and protein level led to changes in the levels and/or properties of lipids of the cells and their lysosomes. These results are of great relevance to understanding the interplay between PD related changes in lipid levels and αS pathology.

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GM1 micelles affect Amyloid Beta aggregation by co-assembly

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Background: Amyloid beta peptide (Aβ) is the key component of extracellular senile plaques in Alzheimer's disease. It has been shown that GM1 ganglioside is co-localized with Aβ in brain plaques,[1] and GM1-containing vesicles can mediate the aggregation of Aβ *in vitro*.[2]

Questions addressed: Q1. Do GM1 lipids co-assemble with Aβ monomers or fibrils *in vitro*? **Q2.** How does this co-assembly affect Aβ aggregation kinetics? **Q3.** What steps of the aggregation process are affected by GM1?

Methods: To answer **Q1**, Microfluidic Diffusional Sizing (MDS) was used to measure the apparent hydrodynamic radius (R_h) of Aβ42 monomers with increasing GM1 concentration, and confocal Microscopy and Cryogenic Electron Microscopy (Cryo-EM) were used to investigate co-assembly of GM1 micelles and Aβ40 and 42 fibrils. To answer **Q2**, P-FTAA fluorescence measurements have been used to monitor Aβ40 and 42 aggregation kinetics with varying GM1 concentrations. To answer **Q3**, Aβ aggregation kinetics were studied with 1% and 25% seeds with varying GM1 concentrations.

Results and discussion: Co-assembly of lipids with both Aβ monomers and fibrils is observed. According to the MDS results, the apparent R_h raised from around 2 nm (radius of Aβ42 monomers) to 5 nm (radius of GM1 micelles) with increasing GM1 concentration. This indicates that Aβ42 monomers co-assemble with GM1 micelles. In addition, confocal microscopy images (Figure 1, first row) revealed a co-localization of lipids (red channel) and fibril clusters (green channel). At higher resolution, Cryo- EM images (Figure 1, second row) revealed Aβ fibrils decorated by small objects that are interpreted as GM1 micelles.

The observed co-assembly may influence Aβ aggregation kinetics. Non-seeded kinetic results show that the aggregation of both Aβ40 and 42 is accelerated at low GM1 concentration. While Aβ40 aggregation is delayed with increasing GM1 concentration, that of Aβ42 is still accelerated. In terms of seeded aggregation reactions, both Aβ40 and 42 aggregations with 1% or 25% seeds are delayed by the addition of GM1. These changes in the aggregation kinetics may be due to GM1 micelles influencing the primary nucleation through interactions with monomers and small oligomers, and inhibition of elongation and secondary nucleation by GM1 micelles blocking the ends and sides of fibrils.

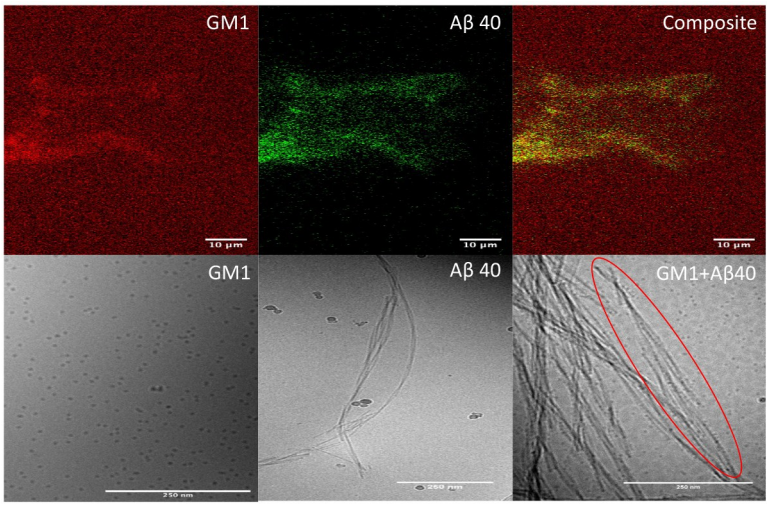

Figure 1. First row: confocal microscopy images of GM1 and Aβ40 at lipid/protein molar ratio of 160. GM1 is labeled with Atto-DOPE (red). Aβ40 is labeled with P-FTAA (green). All scale bars = 10 µm. Second row: Cryo-EM images of GM1 alone, Aβ 40 alone, and the mixture of GM1 and Aβ40 at molar ratio of 160. The red circle encloses cases where lipids are decorating Aβ fibrils. All scale bars = 250 nm.

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Atomic Resolution Insights into pH Shift Induced Deprotonation Events in LS-shaped Aβ(1-42) Amyloid Fibrils

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Background: Alzheimer's disease (AD) is a neurodegenerative and progressive neurological disorder and associated with the deposition of misfolded aggregates of the amyloid-β protein (Aβ). Aβ(1-42) is one of the most aggregation-prone components in senile plaques of AD patients. In the past we demonstrated that relatively homogenous Aβ(1-42) fibrils with one predominant fold visible in solid-state NMR-spectra can be obtained at acidic pH.[1] The structure of these fibrils resolved at near atomic resolution by cryoEM differs remarkably from some other polymorphs obtained *in vitro* at neutral pH or from *ex vivo* material analyzed so fa [r. In particular, the entire N-terminal region is part of the rigid fibril core. The first 21 residues form two extended β-strands, part of the LS type fold of the monomers.[1]

Questions addressed: For biophysical investigations such as interaction studies with physiological and diagnostic binding partners, reliable and reproducible *in vitro* generation of Aβ(1-42) fibrils with a high degree of homogeneity and known structure is desirable. However, such studies should be conducted under physiological conditions, i.e., at pH values above 4. Thus, knowledge about the stability and structure of this fibril type at high pH values is essential for exploiting the high homogeneity of these well-characterized monomorphic LS fibrils, which are not obtained by fibrillization at higher pH values.

Methods: Here, we probe the stability and morphology of those low-pH Aβ(1-42) fibrils over the investigated pH range from 2 to 7. The protonation state of titratable groups and the stability were investigated at atomic resolution by solid-state NMR-spectroscopy, molecular dynamics (MD) simulations, and standard biophysical techniques, including AFM and CD.

Results and discussion: Here, we show that the global structure of the Aβ(1-42) fibril remains unchanged when grown at acidic pH and adjusted to neutral pH values afterward. However, upon pH adjustment, local changes in the amino acid protonation states occur that affect intra-subunit (H6/E11/H13) and inter-subunit (D1/K28/A42) interactions relevant for the conservation of the fibril structure (summarized in Fig. 1). Performing a pH titration, we observe local changes for pH 6 and more pronounced for pH 7. In Aβ(1-42) fibrils at pH 5 both monomer states are populated. In summary, our study shows that the Aβ(1-42) fibril grown at acidic pH[1] conserves its structure and remains stable at physiological pH conditions. A particularly interesting observation is that the proton exchange of C-terminal A42 is slow on the NMR time scale, whereas for solvent exposed carboxyl groups fast exchange is observed, and we present to our knowledge the first estimation of a pKa value of a C-terminal carboxyl group in an amyloid fibril. Finally, the data qualify this Aβ(1-42) fibril polymorph as a perfect object for for binding and interaction studies over the full pH range from pH 2 to pH 7.

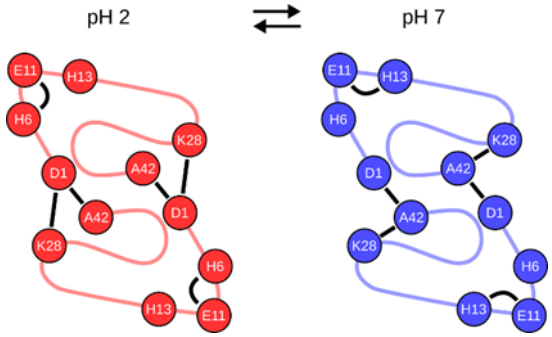

Figure 1. Model of interactions essential for fibril stability at acidic and neutral pH. Schematic of the Aβ(1-42) fibril investigated in the present study (pH conditions shown above). Amino acids identified to be essential for structural stability and relevant salt bridges between them are marked.

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