

Proceedings of the
**International Symposium on
Pathomechanisms of Amyloid Diseases**



Chairs:

Varan Govind (University of Miami)

Ayyalusamy Ramamoorthy (University of Michigan)

University of Miami, December 18-20 2019

International Symposium on Pathomechanisms of Amyloid Diseases

December 18-20, 2019, Miami, Florida, USA

Venue: Donna Shalala Student Center, University of Miami, 1330 Miller Drive, Miami FL 33146
(<https://amyloiddiseases.wixsite.com/symposium2019>)

Schedule

Day-1: December 18, 2019 (Wednesday)

8:30 – 8:55 AM **Meet and Greet** (Coffee and a light breakfast is provided)

8:55 – 9:00 AM **Opening Remarks** (Ayyalusamy Ramamoorthy)

9:00 - 10:40 AM Session I: In-vivo studies

Chairs: Drs. Birgit Strodel and Martin Muschol

- **Varan Govind**, University of Miami, FL
Whole-brain proton MR spectroscopic imaging and diffusion kurtosis imaging in Alzheimer's disease
- **Michal Toborek**, University of Miami, FL
Amyloid pathology at the neurovascular unit
- **Rong Grace Zhai**, University of Miami, FL
Are all aggregates created equal? Probing the biochemical and cellular properties of aggregates *in vivo*
- **David Kang**, University of South Florida, FL
Mitochondrial dysfunction and proteostasis in neurodegenerative disease
- **Jin Hyung Lee**, Stanford University, CA
Innovating high-resolution novel imaging approaches to elucidate mechanisms of prion-like spreading of neurodegenerative disease

10:40 – 11:00 AM **Coffee Break**

11:00 AM - 12:40 PM Session II: Biology and Monitoring of Amyloid Formation

Chairs: Drs. Delia Gabrera and Varan Govind

- **James Bardwell**, University of Michigan, MI
SERF, a tiny well Conserved Protein accelerates Amyloid Formation via Fuzzy Complex Formation
- **Gal Bitan**, UCLA, CA
Biomarker analysis in brain-derived exosomes for improved diagnosis and progression-monitoring of neurodegenerative diseases
- **Magdalena Ivanova**, University of Michigan, MI
Are the bio-molecular traits of protein aggregation and neurodegeneration related?
- **Guillermo Herrera**, University of South Alabama, AL
Glomerular and vascular amyloidogenesis: The role of mesangial cells and pericytes in al-amyloidosis
- **Jerry Yang**, University of California, CA
Can fluorescent probes aid in antemortem diagnosis of amyloid-associated diseases?

12:40 – 1:30 PM **Lunch Break**

1:30 - 3:10 PM Session III: Cellular Studies on Amyloids

Chairs: Drs. Magda Ivanova and Ralf Langen

- **Yanzhuang Wang**, University of Michigan, MI
Where is the toxicity of A β in cells?
- **Olga Gursky**, Boston University, MA
Structural basis for action of Serum Amyloid A protein in lipid transport and immune response
- **Katsumi Matsuzaki**, Kyoto University, Japan
How does A β self-aggregate on and exert toxicity against neuronal cells?
- **Jennifer Lee**, NIH, MD
Is it amyloid? How do you determine secondary structure in cells?
- **Chunyu Wang**, Rensselaer Polytechnic Institute, NY
Rare 3-O-sulfation of heparan sulfate enhances tau interaction, cellular uptake and seeding

3:10 – 3:30 PM Coffee Break

3:30 - 4:50 PM Session IV: Pathology of Amyloids

Chairs: Drs. Jennifer Lee and Devarajan Thirumalai

- **Rakez Kaye**d, University of Texas, TX
Amyloid oligomers cross-seeding; How specific is it? What potential implications for therapeutic approaches?
- **Ralf Langen**, University of Southern California, CA
What is the mechanism of Huntington misfolding? Can we develop tools to monitor and inhibit it?
- **Luis del Pozo-Yauner**, University of South Alabama, AL
Elucidating the mechanism of immunoglobulin light chain amyloid aggregation
- **Christian Camargo**, University of Miami, FL
A clinician's conundrum: The role of Amyloid in defining Alzheimer's disease

4:50 – 5:50 PM Short Presentations (7 min + 3 Q&A for each)

Chairs: Drs. Giuseppe Melacini and Carmelo LaRosa

- **Rashik Ahmed**, McMaster University, Canada
Suppression of Alpha Synuclein Membrane Toxicity by an Extracellular Chaperone
- **Anukool Bhopatkar**, University of Southern Mississippi, MS
Granulins modulate liquid-liquid phase separation and aggregation of TDP-43 C-terminal domain
- **Hanh Dao**, Ohio State University, OH
Structural Studies of GSS-Associated Y145Stop Prion Protein Amyloids by Solid-State NMR Spectroscopy
- **Caitlyn Fields**, University of Wisconsin, WI
Animal propensities for diabetes strengthens oligomer hypothesis as shown using 2D IR spectroscopy
- **Kelly Young**, University of Michigan, MI
Thiol-mediated and catecholamine-enhanced multimerization of a cerebrovascular disease enriched fragment of NOTCH3
- **Arjun Watane**, University of Miami, FL
Investigating Retinal Blood Flow Characteristics and Amyloid Formation in Patients with Type 2 Diabetes and Mild Cognitive Impairment

Day-2: December 19, 2019 (Thursday)

8:30 AM Coffee and a light breakfast is provided

9:00 - 11:00 AM Session V: Amyloid Aggregation Pathways

Chairs: Drs. Yifat Miller and Gal Bitan

- **Ayyalusamy Ramamoorthy**, University of Michigan, MI
Probing the mechanisms of amyloid-beta fibril formation by NMR
- **Stephen Meredith**, University of Chicago, IL
Brain-seeded A β oligomers and fibrils
- **Martin Muschol**, University of South Florida, FL
Origin of amyloid oligomers and their effects on fibril formation
- **Vijay Rangachari**, University of Southern Mississippi, MS
How do lipid interfaces affect A β oligomer generation?
- **Martin Zanni**, University of Wisconsin, WI
Why are the populations of amyloid oligomers so high? And how we might use that information to design a mouse model?
- **Shai Rahimipour**, Bar-Ilan University, Israel
Can we target A β oligomers for early diagnosis of Alzheimer's disease?

11:00 – 11:20 AM Coffee Break

11:20 AM - 1:00 PM Session VI: Structural Insights on Amyloid Aggregation

Chairs: Drs. Antoinette Killian and Ayyalusamy Ramamoorthy

- **Bernd Reif**, Technical University of Munich, Germany
How do small molecules, peptides and chaperones interfere with protein aggregation? - An NMR perspective
- **Markus Zweckstetter**, Max Planck Institute, Germany
How does disease phenotype correlate with protein structure?
- **Kendra Frederick**, UT Southwestern University, TX
Which amyloid conformations are propagated in cells?
- **Witold Surewicz**, Case Western Reserve University, OH
Amyloid fibrils from the N-terminal prion protein fragment as a model for studying the mechanism of prion propagation and transmissibility barriers
- **Antoine Loquet**, University of Bordeaux, France
What are the structural determinants of amyloid cross-seeding

1:00 – 02:00 PM Lunch Break (followed by) **Amyloid Quiz**, Chair: James Bardwell

2:00 - 3:20 PM Session VII: Theoretical and Computations studies

Chairs: Drs. Kendra Frederick and Bernd Reif

- **Devarajan Thirumalai**, University of Texas, Austin, TX
Role of water in protein aggregation and amyloid polymorphism
- **Birgit Strodel**, Institute of Complex Systems, Germany
Amyloid aggregation simulations: what can we learn from them?
- **Yifat Miller**, Ben-Gurion University, Israel
The interplay between metals, peptides and amyloids: mechanisms and therapeutic strategies
- **Prem Chapagain**, Florida International University, FL
Theoretical and computational analysis of the amyloid aggregation kinetics

3:20 AM - 4:40 PM Short Presentations (7 min + 3 min Q&A for each)

Chairs: Drs. Olga Gursky and Stephen Meredith

- **Sidney Dicke**, University of Wisconsin, WI
Extra-islet amyloid plaques found in pancreas of transgenic mice and humans using 2DIR microscopy
- **Pu Duan**, Massachusetts Institute of Technology, MA
Tau Amyloid Fibrils Structure and Dynamics from Solid-State NMR
- **Shon Koren**, University of Florida, FL
Tau drives translational selectivity by interacting with ribosomal proteins
- **Olga Press-Sandler**, Ben-Gurion University of the Negev, Israel
Investigating distinct primary nucleation of A β 1-42 polymorphic dimers
- **Bikash Sahoo**, University of Michigan, MI
Dual Action of Nanodiscs on Amyloid Aggregation
- **Fulvia Verde**, University of Miami, FL
Conserved NDR kinase controls RNP granule assembly to regulate cell growth and chronological lifespan
- **Yiqun Zhou**, University of Miami, FL
Development of Nontoxic Carbon Dots and Their Applications in the Amyloid Inhibition
- **Yi Zhu**, University of Miami, FL
Isoform-specific protection of NMNAT against Tau-induced neurodegeneration by suppressing pathological pTau aggregation

4:40 AM - 5:20 PM Session VIII: Round Table Discussion

- Dr. Claes Wahalestedt (Chair)
- Christian Griesinger
- Danilo Milardi
- Rakez Kayed

7:00 PM Dinner (by invitation)

Day-3: December 20, 2019 (Friday)

8:30 AM Coffee and a light breakfast is provided

8:50 - 10:40 AM Session IX: Dedication to Professor Sir Christopher Dobson

Chairs: Drs. Daniel Raleigh and Marcus Faendrich

- **Marcus Fändrich**, Ulm University, Germany
Analysis of amyloid fibril structures by cryo-EM
- **Alfonso De Simone**, Imperial College, UK
Are functional and pathological forms of alpha-synuclein so different?
- **Astrid Gräslund**, Stockholm University, Sweden
Amyloidogenic nanoparticles in blood serum of patients with Alzheimer's disease
- **Daniel Raleigh**, Stony Brook University, NY
Islet amyloid induced β -cell death from molecular biophysics to therapeutic applications
- **Ehud Gazit**, Tel-Aviv University, Israel
What is the role of metabolites in amyloid disease?

10:40 – 11:00 AM Coffee Break

11:00 AM - 12:20 PM Session X: Amyloid Aggregation related to Type-2 Diabetes

Chairs: Drs. Astrid Gräslund and Martin Zanni

- **Andisheh Abedini**, Stony Brook University, NY
Islet amyloid polypeptide cytotoxicity directly correlates with amyloidogenicity
- **Lucie Khemtémourian**, Université Pierre et Marie Curie, France
What is the role of insulin on the amyloid aggregation of human islet amyloid polypeptide and its mutants?
- **Antoinette Killian**, Utrecht University, Netherlands
Can inhibitors of IAPP aggregation act via a different mechanism depending on whether or not membranes are present?
- **Carmelo La Rosa**, University of Catania, Italy
The lipid-chaperone hypothesis: May amylin, α -synuclein and A β have a common molecular mechanism for membrane damage?

12:20 – 1:00 PM Lunch Break

1:00 AM - 2:00 PM Session XI: Novel Tools to Study Amyloids

Chairs: Drs. Sandrine Ongerer and Vijay Rangachari

- **Yusuke Nishiyama**, JEOL Resonance & RIKEN, Japan
Electron and NMR crystallography to probe amyloid fibers
- **Sunil Saxena**, University of Pittsburgh, PA
Can studying interaction of metal-ion with amyloid- β impact development of ESR spin labeling?
- **Takahiro Watanabe-Nakayama**, University of Kanazawa, Japan
Do the individual amyloid aggregates follow the prion model?

2:00 AM - 4:20 PM Session XII: Amyloid Inhibitors and Potential Therapy

Chairs: Drs. Andisheh Abedini and EHUD Gazit

- **Christian Griesinger**, Max Planck Institute, Germany
Interference into aggregation with small molecules
- **Giuseppe Melacini**, McMaster University, Canada
What is the molecular basis of A β assembly toxicity?
- **Rajeev Prabhakar**, University of Miami, FL
Are computational techniques useful in designing of drugs for amyloid diseases?

Coffee Break

- **Sandrine Ongerer**, Université Paris Saclay, France
What secondary structure should be mimicked in peptide derivatives to inhibit the aggregation of amyloid proteins? Helix or β -hairpin?
- **Bhubaneswar Mandal**, Indian Institute of Technology Guwahati, India
Engineered peptides for total degradation of Amyloid
- **Danilo Milardi**, National Research Council, Italy
Multitarget proteostasis rescuers: a promising venue in amyloid therapy?

Plans for the Future Meetings

Awards (Dr. Olga Gursky)

Closing Remarks (Varan Govind)

Whole-brain 1H-MR Spectroscopic Imaging and Diffusion Kurtosis Imaging in Alzheimer's Disease

Teddy Salan¹, Sulaiman Sheriff¹, Barry Baume² and Varan Govind¹
Departments of Radiology¹ and Neurology², University of Miami, Miami

Purpose: To develop an *in vivo* magnetic resonance imaging protocol for measurements of regional metabolite concentrations, free water fractions, diffusion kurtosis parameters of tissue structures and white-matter lesion volumes at the whole brain level in individuals with Alzheimer's disease (AD).

Methods: MRI data were acquired using a 3 Tesla MRI scanner (Siemens Skyra; 123 MHz for 1H) with a 20-channel head receiver coil for detection. We used an hour-long MRI protocol that consists of whole-brain MRSI, diffusion kurtosis imaging (DKI), T1-, FLAIR- and T2*-MRI sequences. MRSI data were acquired using a volumetric spin-echo echo-planar spectroscopic imaging (3D-EPSI) sequence (TR1: 1551 ms, TR2: 511 ms; TE 17.6 ms, 135 mm slab, field-of-view (FOV): 280x280x180 mm³, 100x50x18 spatial points and an acq. time of 17 minutes; Figure 1). DKI data acquisition used a 2D diffusion-weighted spin-echo echo-planar dual-shell imaging sequence ($b=1000, 2000$ s/mm²) with 30 diffusion gradient directions per shell (TR: 9000 ms, TE 89 ms, 2.5 mm³ isotropic voxel), and 2 non-diffusion-weighted acquisitions ($b=0$ s/mm²) with opposite phase-encoding directions. Using this protocol, we acquired data from two subjects with AD (female, 77 years; male, 79 years).

We processed EPSI datasets using the MIDAS package.¹ Briefly, imported data went through a pipeline of processing steps. These included formation of volumetric metabolite images, co-registration of T1-MRI and MRSI, segmentation of T1-MRI, calculation of MRSI voxel tissue content, signal normalization using tissue water reference signal, and spatial registration to the Montreal Neurological Institute (MNI) single-subject MRI template.² For spectral fitting, brain metabolite prior information for N-acetyl aspartate (NAA), total-creatine (Cr), total-choline (Cho), myo-inositol (m-Ins), and glutamate plus glutamine (Glx) were simulated using an in-house developed program that used the GAMMA library³ and published chemical shifts and coupling constants.⁴ The nominal MRSI voxel volume as acquired was ~0.3 mL. After zero-padding data to 64x64x32 matrix and applying Gaussian spatial smoothing in the three orthogonal directions, the voxel volume used for data analysis was ~1 mL.

DKI data processing steps follow the pipeline depicted in Figure 2. 1) The diffusion-weighted (DW), FLAIR, and T1 images were first pre-processed using FSL.⁵ DW images are corrected for geometric, eddy current, and susceptibility-induced distortions by applying *Eddy* and *Topup* functions on the opposite phase-encoded $b=0$ pairs. 2) The corrected DW images are skull-stripped with *BET*. 3) FLAIR and T1 images were coregistered using FLIRT then the FLAIR images were skull-stripped with *BET*. 4) T1-MRIs were segmented using *FAST* to obtain a white matter (WM) mask. 5) We obtained a white matter hyperintensity (WMH) lesion mask from FLAIR images using an appropriate intensity threshold method. 6) After pre-processing, DKI fitting was performed using the *DIPY* library⁶, with additional in-house developed software to obtain free water volume fraction (f_{FW}) and free-water eliminated (FWE) metrics⁷ such as fractional anisotropy (FWE-FA) and mean kurtosis (FWE-MK). In addition, we calculated FA and MK using the conventional free-water

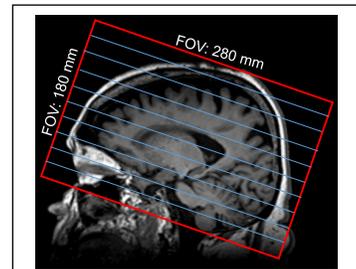


Figure 1: Whole-brain 3D-EPSI – the brain coverage.

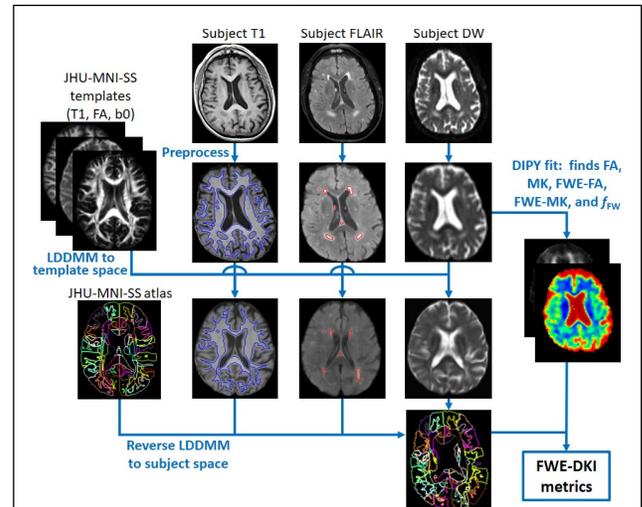


Figure 2: Pipeline of FWE-DKI processing with atlas analysis. After pre-processing and extracting WM and WMH maps, the T1, FLAIR, and DW-images are transformed to the JHU-MNI-SS 'Eve' template space. FWE-DKI fitting is performed on the DW-data in subject space to obtain parametric maps. We obtained data from atlas regions for analysis by reverse transformation of the WM, WMH, and atlas images to the subject space.

non-eliminated method. 7) For spatial registration of all images to a template MRI on which an anatomically labelled brain regional atlas is created (i. e, JHU-MNI-SS), we used the *LDDMM* method⁸ available within the *DiffeoMap* program. 8) Finally, we spatially inverse transformed all images to the original subject space. DKI metrics data obtained in the subject space images were used for analysis.

Results: Whole-brain MRSI datasets obtained from two subjects indicate that spectra from ~58% of voxels within the brain volume met stringent spectral quality criteria set for quantitation purposes. We typically obtain good quality spectra from ~ 65-70 % voxels within the brain of healthy control subjects. The reduced % of good quality spectra in the brains of AD subjects is due to significantly reduced brain tissue volume in AD. Increased m-Ins and Cho, and decreased NAA were noted in regions containing WMH lesions (Figure 3).

Our FWE-DKI processing method allows us to calculate both the conventional (FA and MK) and FWE (FWE-FA, FWE-MK) diffusion metrics. Figure 4 shows comparisons of measures obtained from two regions containing WMH (i.e., the right corona radiata and right pre-central gyrus) and the same regions from the contralateral left side. Our results indicate increased free water fraction (f_{FW}), and decreased FA, FWE-FA, MK, and FWE-MK in the regions containing WMH lesions.

Discussion: Previous studies^{9,10} have reported reduced NAA and elevated Cho in WMH⁹ and normal appearing¹⁰ regions in subjects with AD. However, these studies have utilized a single-slice or a multi-slice MR spectroscopic imaging sequence for data acquisitions; both have limitations to collect data from the whole brain. However, we used an in-house developed a 3D-EPSI sequence for acquisition of spectroscopic data from the whole-brain in this study. DKI data acquisition typically includes the whole-brain coverage. Analysis of brain MRI data obtained from subjects with AD and healthy elderly controls poses complications due to the presence of patchy regions of signal hyperintensity on T2-weighted MRIs (e.g., FLAIR) predominantly in deep and periventricular white matter areas. These WMHs are considered as a manifestation of small vessel disease¹¹ and the underlying pathological processes include neuroinflammation,¹² demyelination and axonal loss among others.¹³ In this study, we developed a whole-brain imaging protocol for evaluation of metabolite and microstructural alterations and free-water fractions in WMH and normal appearing anatomical regions within the brain of individuals with AD.

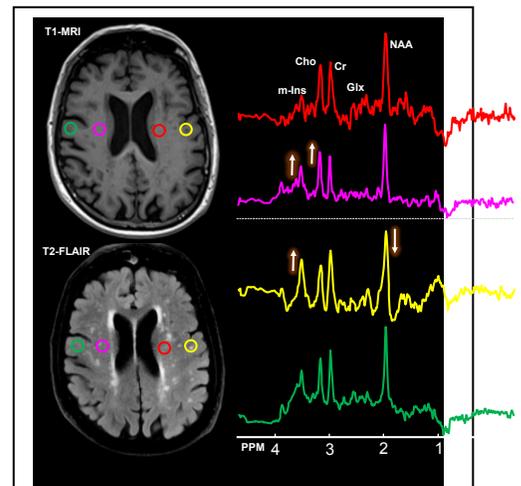


Figure 3: Short-TE 1H-spectroscopic imaging of a slice in the brain of a subject with Alzheimer's disease (Male, 79 years; the slice in axial orientation). T1-MRI, T2-FLAIR and spectra from 4 voxels are shown. The spectra from two voxels on the right side (red and yellow circles) include white matter hyperintensity lesions whereas the spectra from two voxels on the contralateral side (purple and green) do not include any obvious lesions.

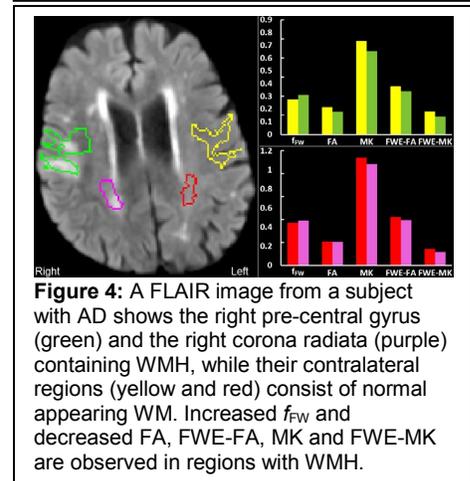


Figure 4: A FLAIR image from a subject with AD shows the right pre-central gyrus (green) and the right corona radiata (purple) containing WMH, while their contralateral regions (yellow and red) consist of normal appearing WM. Increased f_{FW} and decreased FA, FWE-FA, MK and FWE-MK are observed in regions with WMH.

References: [1]. Maudsley AA, Darkazanli A, Alger JR, et al. *NMR Biomed.* 2006;19:492-503. [2]. Collins DL, Zijdenbos AP, Kollokian V, Sled JG, Kabani NJ, Holmes CJ, Evans AC. *IEEE Trans Med Imaging.* 1998;17:463-468. [3] Smith SA, Levante TO, Meier BH, Ernst RR. *Journal of Magnetic Resonance, Series A.* 1994;106:75-105. [4] Govindaraju V, Young K, Maudsley AA. *NMR Biomed.* 2000;13:129-153. [5] Smith SM, Jenkinson M, Woolrich MW, Beckmann CF, et al. *Neuroimage.* 2004;23 Suppl 1:S208-219. [6] Garyfallidis E, Brett M, Amirbekian B, Rokem A, et al.. *Front Neuroinform.* 2014;8:8. [7] Salan T, Sheriff, S., Govind, V. Diffusion kurtosis imaging of the brain with free water elimination. Paper presented at: International Society for Magnetic Resonance in Medicine 2019; Montreal, Canada. [8] Ceritoglu C, Tang X, Chow M, et al.. *Front Neurosci.* 2013;7:151. [9] Constans JM, Meyerhoff DJ, Gerson J, et al. *Radiology.* 1995;197:517-523. [10] Su L, Blamire AM, Watson R, et al.. *Transl Psychiatry.* 2016;6(8):e877. [11] Ter Telgte A, van Leijns EMC, Wiegertjes K, et al. *Nat Rev Neurol.* 2018;14:387-398. [12] Verhaaren BF, Debette S, Bis JC, et al. *Circ Cardiovasc Genet.* 2015;8:398-409. [13] Rosenberg GA, Wallin A, Wardlaw JM, et al. *J Cereb Blood Flow Metab.* 2016;36:6-25.

Extracellular vesicle-mediated amyloid transfer to neural progenitor cells: implications for RAGE and HIV infection

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Aims/Purpose

HIV-infected brains were shown to have increased amyloid beta (A β) deposition. This phenomenon has been linked to the development of cognitive dysfunction based on the observation that early beta-amyloidosis in HIV-1-infected patients was associated with HIV-associated neurocognitive disorders (HAND). A β deposition occurs mostly in the perivascular space, which points to the brain microvessels having a role in amyloid pathology. In support of this notion, the blood-brain barrier (BBB) was postulated to be critical for A β homeostasis as an interface that may contribute to A β accumulation in the brain.

ECVs are recognized as the carriers of biologically active proteins and genetic materials, such as mRNA, microRNA, siRNA, and DNA, which implicate them in the physiology and pathology of the CNS. However, the mechanisms of ECVs generation by the parent cells and the mechanisms of their uptake by the recipient cells are not fully understood. In this work, we examined two critical aspects of the involvement of the receptor for advanced glycation endproducts (RAGE) in the ECV-mediated A β pathology in the context of HIV infection. First, we evaluated the role of RAGE in endothelial cell-derived ECV release and A β -ECV levels. Second, we examined the involvement of RAGE in the ECV-mediated transfer of A β to the neighboring neuronal progenitor cells (NPC) and its effects on neuronal differentiation.

The goals of our studies were to address the hypothesis that A β can be transferred via ECVs from brain endothelial cells to NPCs and that this process can contribute to abnormal NPC differentiation. Mechanistically, we focused on the role of RAGE and activation of the inflammasome in these events.

Methods

Confluent human brain microvascular endothelial cells (HBMEC) were exposed to 30 ng/ml HIV-1 particles and/or 100 nM A β (1-40) (or A β HyLite) for 48 h. Selected cultures were pretreated with 500 nM FPS-ZM1 (RAGE inhibitor) for 2 h. ECVs were isolated from conditioned medium using ExoQuick-TC exosome precipitation solution (System Biosciences) according to the manufacturer's specifications. Human NPCs were exposed to HBMEC-derived ECVs for 24 h, with selected cultures additionally treated with 500 nM FPS-ZM1 (FPS).

ECVs were analyzed by NanoSight model NS300 (Malvern Instruments Company, Nanosight, Malvern, United Kingdom). A β transfer was evaluated by fluorescence and confocal microscopy and ELISA. Protein colocalization was visualized by confocal microscopy and protein expression by immunoblotting and immunofluorescence. NPC differentiation was studied by evaluation of Hu C/D, NeuN, and DCX to assess neurons at different stages of development.

Results

Exposure of HBMEC to HIV and/or A β significantly increased ECVs release and their cargo content, including A β . HBMEC form a functional unit with the surrounding pericytes, perivascular astrocytes, microglia, and neurons, called the neurovascular unit. Therefore, we hypothesized that HBMEC-derived ECV may transfer A β to other cells of the neurovascular unit and neighboring neural progenitor cells (NPCs). Indeed, our published results indicate that ECVs can effectively transfer A β to astrocytes and pericytes. Moreover, ECVs can interact with the blood-brain barrier (BBB) and carry A β across the BBB into the brain parenchyma.

Approximately 47% of dividing progenitor and 46% of transit amplifying cells are located within 5 microns of the brain endothelium. Therefore, we hypothesized ECVs may increase NPC A β exposure and that this process may affect NPC differentiation into mature neurons. In addition, we proposed that RAGE may be a key player in the HIV-induced brain endothelial ECV release and A β -ECVs transfer to NPCs.

Our results indicate that NPC cultures readily taken up A β , especially from ECVs isolated from HIV plus A β -treated HBMEC. This effect was statistically higher as compared to cultures treated alone with A β -ECVs and HIV-ECVs. Similar results were obtained when quantifying A β uptake to the nuclei of the recipient NPCs. RAGE inhibition with FPS on the recipient NPCs significantly reduced nuclear transfer of A β by A β +HIV ECVs.

The inflammasomes are cytoplasmic complexes containing the danger signal sensing proteins of the NLR family, such as NLRP3 evaluated in the present study. The NLR proteins can then recruit the adaptor ASC (apoptosis-associated speck-like protein containing a caspase recruitment domain) and form cytoplasmic complexes, leading to activation of caspase-1 as well as release of IL-1 β and IL-18. Exposure to HIV-ECV significantly increased the total and nuclear ASC levels. In addition, NLRP3 nuclear levels increased significantly after HIV+A β -ECV exposure as compared to control. These results are consistent with the reported role of inflammasome in HIV infection and amyloid pathology. Nevertheless, no changes in the inflammasome pathway end-players, such as caspase 1 activity and/or IL-1 β levels, were detected after the employed ECV exposure, suggesting that the classical inflammasome pathway was not induced in NPCs. RAGE inhibition modulated levels and cellular localization of both ASC and NLRP3 in response to A β and/or HIV-ECV exposure, supporting the notion that RAGE can signal through the NLRP3 inflammasome.

We also evaluated the role of HBMEC-derived ECVs on differentiation of NPCs to mature neurons. The rationale of these experiments was enhanced by a report that A β can affect neuronal differentiation via the inflammasome pathway involving RAGE. Cell differentiation was evaluated by counting cells positive for the neuronal marker Hu C/D, neuronal nuclear antigen (NeuN, a marker for mature neurons), and doublecortin (DCX, a marker of immature neurons). The number of Hu C/D-positive cells was markedly diminished by exposure to ECVs derived from A β -treated HBMEC, suggesting impaired neuronal maturation. Interestingly, the number of Hu C/D-positive cells was also significantly lower in the HIV+A β -ECV+FPS group when compared to the HIV+A β -ECV group, indicating that RAGE inhibition in the recipient NPCs diminished neuronal differentiation in response to A β -ECV transfer only in the presence of HIV. The number of NeuN positive cells was not affected by the A β -ECV treatment; however, it was significantly increased in the presence of HIV-ECV as compared to the control. RAGE inhibition significantly increased NeuN positive cell number in the A β -ECV+FPS group as compared to the A β -ECV group. The number of NeuN/DCX double positive cells did not change significantly as the result of the employed treatment factors. Overall, these results suggest that A β -ECV, HIV-ECV, and/or RAGE inhibition have a modulatory effect on neuronal differentiation.

Discussion

ECVs are recognized as the carriers of biologically active proteins and genetic materials, such as mRNA, microRNA, siRNA, and DNA, which implicate them in the physiology and pathology of the CNS. However, the mechanisms of ECVs generation by the parent cells and the mechanisms of their uptake by the recipient cells are not fully understood.

Novel findings of the present study indicated that inhibition of RAGE in brain endothelial cells resulted in a significant increase in produced ECVs, and a decrease in A β and protein levels in ECVs. These changes appeared to be specific because we did not observe any alterations in ECV number upon nSMase inhibition. Results also indicate that ECVs can transfer A β from the brain endothelial cells to NPCs, and that this process is facilitated in the context of HIV. A β was also delivered to the nuclei of the recipient NPCs, a process that can result in profound transcriptomic changes. Important results indicate that transfer A β from the brain endothelial cells to NPCs induces expression of the inflammasome proteins and affects NPC differentiation.

Conclusion

Our results indicate that ECVs can efficiently transfer A β from brain endothelial cells to the neighboring NPCs. Importantly, this process is influenced by HIV and regulated, at least in part, by RAGE. The long-term effect of A β transfer to NPCs include alterations of NPC differentiation via a mechanism that may involve changes to the inflammasome machinery. Overall, these changes may contribute to the development of neurocognitive impairment and A β pathology in HIV-infected brains.

Are aggregates created equal? Probing the biochemical and cellular properties of aggregates *in vivo*.

Yi Zhu, Chong Li, Xianzun Tao, Jennifer M. Brazill, Joun Park, Zoraida Diaz-Perez, R. Grace Zhai

Department of Molecular and Cellular Pharmacology, University of Miami Miller School of Medicine, Miami, FL 33136, USA

Purpose: Accumulative aggregation of mutant Huntingtin (Htt) is a primary neuropathological hallmark of Huntington's disease (HD). Currently, mechanistic understanding of the cytotoxicity of mutant Htt aggregates remains limited, and neuroprotective strategies combating mutant Htt-induced neurodegeneration are lacking. Here, we set out to characterize the cytotoxicity of mutant Htt aggregation in an *in vivo Drosophila* HD model, and investigate the neuroprotective effect of nicotinamide mononucleotide adenylyltransferase (Nmnat), an evolutionarily conserved nicotinamide adenine dinucleotide (NAD⁺) synthase in HD.

Methods: To establish a HD model in *Drosophila*, we used a pan-neuronal driver *elav-GAL4* to express monomeric red fluorescent protein (mRFP)-tagged 588 aa N-terminal fragment of human Htt with either nonpathogenic 15 polyQ expansion or pathogenic 138 polyQ expansion in *Drosophila* (1). We comprehensively characterized the age-dependent neurodegenerative phenotypes induced by expression of mutant Htt with 138Q expansion and investigated the cellular and biochemical process of mutant Htt aggregation at neuronal subcellular compartments. To explore the therapeutic potential during a more clinically relevant condition where mutant Htt protein has been accumulating for some time upon HD diagnoses, we employed a dual-binary expression system *QF/QUAS* and *GAL4/UAS* to temporally separate the expression of Htt and Nmnat. Specifically, we used *nsyb-QF2*, a pan-neuronal driver, to express wild-type or mutant Htt exon 1 translated fragment Q25 or Q91, respectively, from embryonic stage following the promoter of synaptobrevin (2); we then used *elav-geneswitch (GS)-GAL4*, to induce neuronal Nmnat overexpression by mifepristone (RU486) feeding at different time points post phenotypic onset (3).

Results: We show that in *Drosophila* models of HD, the quantity of neuronal compartment-specific accumulation of mutant Htt aggregates correlates with the severity of neurodegenerative phenotypes. Additionally, we uncover an age-dependent acquisition of thioflavin S-positive, amyloid-like adhesive properties of mutant Htt aggregates as the underlying neurotoxicity. We find that neuronal Nmnat expression significantly mitigates mutant Htt-induced neurodegeneration by reducing mutant Htt aggregation through promoting autophagic clearance. Importantly, Nmnat reduces progressive accumulation of amyloid-like Htt aggregates and inhibits the interaction of mutant Htt with mitochondria and essential proteins, including active zone matrix protein, membrane protein, and cytoskeletal protein, in distinct neuronal compartments, thereby restoring synaptic and neuronal function. Conditional expression of Nmnat after the onset of degenerative phenotypes delays the progression and reduces the severity of neurodegeneration at different disease stages, revealing the therapeutic potential of Nmnat-mediated neuroprotection.

Conclusion: Our study uncovers mechanistic insights to the neurotoxicity of mutant Htt aggregation and describes the molecular basis of Nmnat-mediated neuroprotection in HD.

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Role of TDP-43 and CHCHD10 in Mitochondrial Dysfunction and Proteinopathy via Common Mechanisms

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Abstract:

Frontotemporal lobar degeneration (FTLD) associated frontotemporal dementia (FTD) and amyotrophic lateral sclerosis (ALS) are progressive neurodegenerative conditions sharing multiple common genetic and pathological links. The FTLD-TDP variant comprising ~50% of FTLD and >95% of ALS are pathologically characterized by the accumulation of TDP-43 pathology, which are typically seen as aggregated cytoplasmic TDP-43 inclusions mislocalized from the nucleus. These cases invariably exhibit severe mitochondrial abnormalities, which are closely associated with TDP-43 pathology. Indeed, recent studies indicate that the accumulation of TDP-43 in mitochondria of neurons induces mitochondrial dysfunction and synaptic damage.

The cristae structure of mitochondria is formed from the folds created by the inner membrane, which provides an extended surface area allowing the localization of OXPHOS enzymes to produce Adenosine triphosphate (ATP). The maintenance of cristae structure is regulated by an important complex called mitochondria contact site and cristae organization system (MICOS), which is concentrated at cristae junctions, contact sites at the neck of inner membrane folds. MICOS is a large protein complex containing several mammalian subunits including Mitofilin/Mic60, CHCHD3/Mic19, CHCHD6/Mic25, APOO/Mic23/Mic26, APOOL/Mic27, QIL1/Mic13 and MINOS1/Mic10. Mitofilin/Mic60, the key component of MICOS and an inner membrane protein, is an inner membrane protein essential for maintaining mitochondrial cristae structure and respiration as well as cellular viability in response to stress, as the loss of mitofilin not only disrupts cristae structure and respiration but also deregulates mitochondrial fission/fusion, protein import, and mtDNA stability/transcription. Mitofilin also interacts with Optic atrophy 1 (Opa1), an inner membrane protein best known for its role in mitochondrial fusion, and interaction between mitofilin and Opa1 interaction has been shown to be key in regulating inner membrane fusion.

The nuclear gene *coiled-coil-helix-coiled-coil-helix domain containing 10 (CHCHD10)* encodes a small 15-kDa mitochondrial protein, which is mutated in sporadic and familial FTD-ALS spectrum disorders, implicating the significant role of mitochondria in FTD/ALS. *CHCHD10* mutations are also associated with Charcot-Marie-Tooth disease type 2 (CMT2), mitochondrial myopathy, and spinal muscular atrophy Jokela type (SMAJ). *CHCHD10* is recognized as an important mitochondrial protein regulating mitochondrial respiration as well as maintenance of mitochondrial genome and cristae structure. Previous studies identified *CHCHD10* as a component of MICOS, in which *CHCHD10* physically interacts with the core protein mitofilin, and fibroblasts derived patients carrying the *CHCHD10* S59L mutation exhibit impaired MICOS and disrupted cristae structure compared to healthy controls. In our previous study, we discovered a significant connection between FTD/ALS-linked R15L and S59L *CHCHD10* mutations and TDP-43 cytoplasmic localization, which often mislocalized to mitochondria. Wild type *CHCHD10*, on the other hand, played a protective role against TDP-43 in mitochondrial and synaptic integrity. However, several important questions remain to be answered. First, what is the role of

endogenous CHCHD10 in FTLD-TDP? Second, how do CHCHD10 mutations and TDP-43 produce mitochondrial abnormalities and are they through common mechanisms? Third, how does wild type CHCHD10 protect against TDP-43-induced mitochondrial damage? Finally, do CHCHD10 variants regulate the proteostasis and aggregation of CHCHD10 and TDP-43 in mitochondria?

To address these questions, we employed human brain tissues from control and FTLD-TDP cases, TDP-43 transgenic mice, transfected cells, and CHCHD10 transgenic mouse variants utilizing a variety of cellular and biochemical methods. A previous study reported CHCHD10 as a component of MICOS, interacting with the core component mitofilin. However, the functional role of endogenous CHCHD10 in MICOS complex is unknown. Indeed, we confirmed the presence of the endogenous mitofilin-CHCHD10 complex by proximity ligation assays (PLA) In transfected cells, RNAi-mediated knockdown of CHCHD10 destabilized MICOS components, including mitofilin/mic60, mic23/26, and mic19. Blue native gel analysis confirmed the reduction of native high molecular weight mitofilin MICOS complexes secondary to CHCHD10 knockdown, demonstrating that endogenous CHCHD10 is essential for maintenance of MICOS. In addition to other MICOS components, mitofilin also interacts with Optic atrophy 1 (Opa1), an inner membrane protein best known for its role in mitochondrial fusion. The name Opa1 is derived from dominant mutations in the *Opa1* gene linked to inherited optic neuropathy, hearing loss, ataxia, sensorimotor neuropathy, and mitochondrial myopathy in humans. Assessment of native Opa1 complexes showed the presence of Opa1 in the same ~720 kDa complex as mitofilin, and CHCHD10 knockdown significantly reduced this native Opa1 complex as effectively as the mitofilin complex. In contrast to knockdown of endogenous CHCHD10, overexpression of FTD/ALS-linked R15L or S59L CHCHD10 mutations did not destabilize MICOS components but rather significantly disrupted the high molecular weight mitofilin complex as well as the complex between Opa1 and mitofilin, which were confirmed in CHCHD10 R15L and S59L transgenic mouse brains. Such disruption of Opa1-mitofilin complex by R15L and S59L mutations resulted in impaired mitochondrial fusion in photoactivatable mito-drendra2 fusion assays and impaired mitochondrial respiration in Seahorse OCR assays. In human FTLD-TDP brains, we observed a significant reduction of CHCHD10 protein and native high molecular weight mitofilin and Opa1 complexes, both of which were confirmed in TDP-43 transgenic mouse brains. Hence, TDP-43 overexpression resulted in significant reduction in Opa1-mitofilin complexes, impaired mitochondrial fusion, and reduction in mitochondrial respiration, all of which were rescued by wild type CHCHD10. In transfected cells and transgenic mouse brains, we observed a greater propensity for CHCHD10 mutations to become insoluble and aggregate, and proteostasis experiments utilizing TDP-43 import to isolated mitochondria demonstrated that the S59L mutation promotes insolubility and aggregation of both CHCHD10 and TDP-43 in mitochondria. These findings taken together support the notion that TDP-43 or CHCHD10 deregulation produce mitochondrial dysfunction and proteinopathy at least partially through common overlapping mechanisms in FTLD-TDP.

Is It Amyloid? How Do You Determine Secondary Structure in Cells?

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Aims. The overarching goal of this work is to develop spectroscopic probes and methodologies to observe and evaluate protein conformational changes in a cellular environment. We aim to measure secondary structural and site-specific changes that occur upon protein-lipid interactions, amyloid fibril formation, and proteolytic processing. Although methods for study of amyloid formation *in vitro* are robust, the small size and unstructured nature of many of the amyloidogenic proteins present a significant challenge in the complex cellular milieu. Understanding how and where amyloid formation occurs in a cell will provide new therapeutic targets which are desperately needed for this process that is implicated in over 30 human diseases, some of the most notable of which are Alzheimer's and Parkinson's. Further, investigations into the proteolytic fate and degradation mechanisms of misfolded and aggregated proteins will elucidate how proteostasis goes awry, leading to pathogenesis.

Methods. We have chosen Raman microspectroscopy as a method to interrogate amyloid fibril structures in cells. This is a simple, elegant technique as it is easy to couple a monochromatic laser light source and a Raman spectrometer to an inverted microscope for cellular imaging. Furthermore, measurements in water, the integral solvent in cellular environments, are possible due to its weak scattering in the pertinent spectral regions such as the polypeptide amide backbone vibrations (C=O/C-N). To gain spectral contrast against endogenous Raman signals, we are utilizing isotopic ^{13}C -labeling to shift amide-I bands, which inform on the secondary structural changes, and unnatural amino acids such as alkyne-containing (C \equiv C) homopropargylglycine (HPG) and 4-ethynyl-phenylalanine (F_{CC}) for distinguishing spectroscopic signals in the cellularly quiet region $\sim 2100\text{ cm}^{-1}$. Through biosynthetic incorporation, native Met-residues in recombinant proteins can be substituted with HPG in a Met-auxotrophic *E. coli* strain. For F_{CC}, an evolved aminoacyl tRNA synthetase/tRNA pair ("21st pair technology") is used for site-specific incorporation at an engineered amber stop codon site and can be performed in either *E. coli* or cultured mammalian cells. The terminal alkyne moiety also enables the possibility of bioorthogonal chemistry to "click" on fluorogenic molecules for complementary imaging modalities as well as affinity tags such as biotin to perform pull-down assays and proteomic analysis.

Results. Towards our goals, we have focused our efforts on studies on α -synuclein (α -syn), an amyloidogenic protein involved in Parkinson's disease (PD), Multiple System Atrophy, and Lewy body dementia. α -Syn is most well-known for its involvement in the etiology of PD, where α -syn amyloid fibrils are found in Lewy bodies (LB), a histopathological hallmark of the disease. Membrane association of α -syn is associated with its biological function and is implicated in pathogenesis. Upon membrane association, α -syn adopts an α -helical structure, whereas the protein is disordered in solution. In a disease state, β -sheet-rich amyloid fibrils of aggregated α -syn accumulate in the cytosol. Thus far, we have used Raman microspectroscopy to study α -syn conformational changes from soluble to lipid micelle-bound and aggregated amyloid forms. Fibrils formed by PD-related variants including early-onset PD mutants (A30P, E46K, G51D, and A53T)¹ and LB-derived C-terminal truncations (1–103 and 1–122)² have been characterized. Uniformly and segmentally ^{13}C -labeled proteins³ have been utilized to monitor β -sheet formation during amyloid formation. Expression and purification of HPG⁴ and F_{CC}-containing α -syn have been achieved. Importantly, cellular Raman measurements of cultured N27 rat dopaminergic neuronal and SK-MEL-28 human melanoma cells treated with fibrils formed *in vitro* by HPG- and F_{CC}-containing α -syn have been performed, demonstrating that the detection of C \equiv C stretching frequency is feasible.

Conclusion. Raman spectroscopic characterization of α -syn amyloid fibrils formed *in vitro* has shown that both amide-I and amide-III stretching frequencies are sensitive reporters of fibril structural differences as a result of solution conditions and PD-related mutations.¹ This is important because our data would suggest that fibril polymorphs can be formed in different cellular compartments like the acidic lysosomes and potentially result in distinct phenotypes. By using native chemical ligation, we constructed a segmentally isotopically-labeled α -syn, providing region-specific Raman signatures $^{13}\text{C}=\text{O}$ vs. $^{12}\text{C}=\text{O}$ of the polypeptide *during* fibril formation. Mechanistic insights were revealed in which the N-terminal residues were first to develop β -sheet character as a distinctive intermediate; however, it was not until C-terminal residues exhibited β -sheet content that fibril morphology could be visualized by transmission electron microscopy, establishing the importance of both N- and C-terminal regions. Finally, the coupling of terminal alkyne and Raman microspectroscopic measurements of cellular treatments of fibrils formed *in vitro* showed that the presence of fibrils can be identified by their $\text{C}\equiv\text{C}$ stretching frequencies, which exhibited site-specific environmental differences, while maintaining β -sheet structure. Importantly, lipid co-localization and accumulation is revealed, suggesting an intimate relationship between membranes and amyloid toxicity. Collectively, we view that Raman microspectroscopy is a versatile technique that offers structural and molecular-level insights into mechanisms of protein misfolding and amyloid formation as they relate to disease. We hope that our strategy of incorporating bio-orthogonal vibrational probes into proteins will become broadly applicable and contribute to further cellular investigations in the amyloid field.

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A genetic selection to isolate host factors involved in amyloid formation

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Aims: In contrast to the myriad approaches available to study protein misfolding and aggregation *in vitro*, relatively few tools are available for the study of these processes in the cellular context. This is in part due to the complexity of the cellular environment, which, for instance, interferes with many spectroscopic approaches. Here, we describe a tripartite fusion approach that can be used to assess *in vivo* protein stability and solubility in the cytosol of *Saccharomyces cerevisiae*. Budding yeast *Saccharomyces cerevisiae* has proven to be a valuable model organism to study protein folding. It is cost-efficient, easy to genetically manipulate, and amenable to high-throughput screens, and thus provides a facile eukaryotic context for deciphering fundamental molecular processes involved in complex *in vivo* protein misfolding phenomena. In addition, several yeast models have allowed for the screening of cellular and chemical factors affecting the stability or aggregation propensity of disease-associated proteins.

Methods: Our biosensors contain tripartite fusions in which a protein of interest is inserted into antibiotic resistance markers. These fusions act to directly link the aggregation susceptibility and stability of the inserted protein to antibiotic resistance. The tripartite biosensor design comprises the fusion of a protein of interest into the middle of a genetic marker protein (Figure 1a). We hypothesize that if the protein of interest folds well and remains stable, the two fused marker halves will come together, efficiently fold up, and thus confer high levels of antibiotic resistance (Figure 1b). However, if the protein of interest is thermodynamically unstable and therefore prone to proteolysis or aggregation, the entire tripartite fusion will become susceptible to degradation or aggregation, either of which will render it nonfunctional or only partially functional. Therefore, we reason that the antibiotic resistance conferred by the tripartite fusion should serve as a direct readout of the stability and solubility

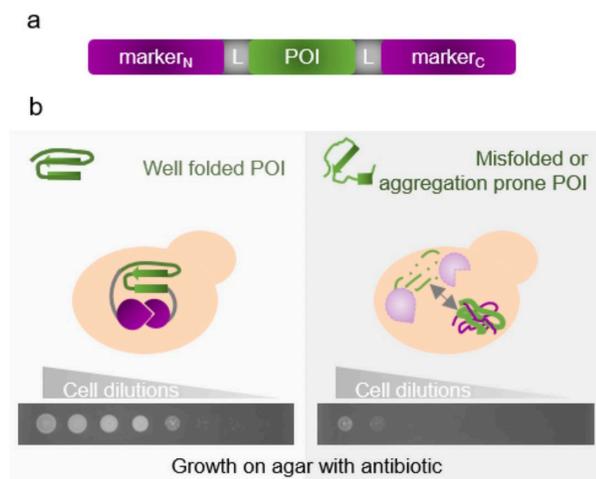


Figure 1

Results: We demonstrate a linear relationship between the thermodynamic stabilities of variants of the model folding protein immunity protein 7 (Im7) fused into the resistance markers and their antibiotic resistance readouts.

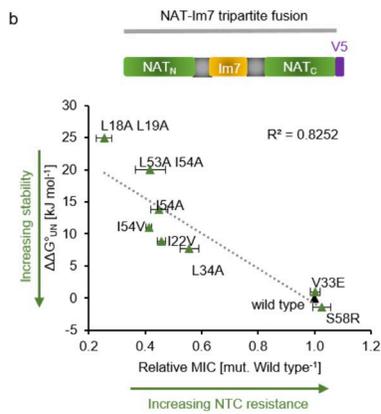
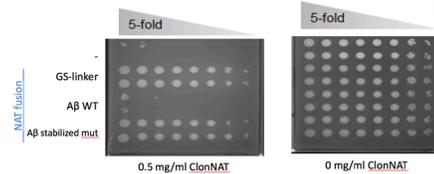


Figure 2: Stability of Im7 variants correlates with antibiotic resistance. Thermodynamically destabilized variants and a stabilized variant of Im7 (S58R) were inserted into the ClonNAT gene via flexible linkers. The level of antibiotic resistance for cells expressing the corresponding fusion constructs was determined as the MIC in serial dilution spot assays

We used this system to investigate the *in vivo* properties of proteins whose aggregation is associated with some of the most prevalent neurodegenerative misfolding disorders, including peptide amyloid beta 1–42 (Aβ42), which is involved in Alzheimer’s disease, and synuclein, which is linked to Parkinson’s disease (Fig 3).

Fig. 3 Cells expressing WT Aβ42 greatly decrease antibiotic resistance of tripartite fusions



Conclusion: Changes in protein stability or solubility play a key role in many fundamental cellular events, such as cell signaling, and are involved in misfolding processes that have been linked to a wide variety of human diseases. In this work, we present a genetic tool to help determine the stability and aggregation propensity of neurodegenerative disease-associated proteins and prion proteins in the yeast cytosol. Applying this tripartite fusion approach to Aβ42 and synuclein, antibiotic resistance appears to depend on other disease-related properties, such as proteolytic sensitivity and the tendency to localize at membranes. Our tripartite system provides a powerful experimental platform that can be used to screen for protein variants, host factors, or small molecules that modulate the disease-related properties of amyloid or prion-prone proteins *in vivo*.

Biomarker analysis in brain-derived exosomes for improved diagnosis and progression-monitoring of neurodegenerative diseases

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Abstract

Goal: The diagnosis of neurodegenerative diseases is difficult due to the lack of reliable biomarkers. The diagnosis typically is made based on clinical presentation, which is highly variable in the patient population leading to a high rate of misdiagnosis. Current biomarker approaches predominantly consist of brain imaging, which is generally expensive and has variable utility in different diseases, and on analysis of cerebrospinal fluid, which is invasive and refused by many patients. Analysis of blood biomarkers has the potential to alleviate these problems, yet due to the efficiency of the blood-brain barrier (BBB), the blood composition does not reflect well biochemical changes in the brain. A potential solution is analysis of biomarkers in brain-derived exosomes, which are shed by brain cells and pass through the BBB into the blood, offering a window into the brain biochemistry using a simple blood draw. In recent years, several groups have demonstrated the utility of this methodology, primarily in the dementia field, whereas progress in other neurodegenerative diseases has been moderate. In the project presented here, we asked if biomarker analysis in brain-derived exosomes could be used to improve the diagnosis of synucleinopathies. In particular, we examined to what extent such an analysis could distinguish Parkinson's disease (PD) and multiple system atrophy (MSA) from healthy individuals and separate these two diseases, which show substantial symptom overlap, especially at early stages, could be separated from each other. In addition, we also asked if serum samples collected postmortem with a short postmortem interval could be used to validate the clinical diagnosis.

Methods: Serum/plasma samples from living persons were obtained from healthy individuals, patients with PD, and patients with MSA in multiple clinics and biobanks and divided into a discovery cohort comprising 50 control, 50 PD, and 30 MSA samples, respectively, and a validation cohort including 50 samples in each group. A postmortem validation cohort comprised 50 PD and 11 MSA samples. Neuronal and oligodendroglial exosomes were isolated from each sample by a 2-step protocol, including first polymer-assisted isolation of all extracellular vesicles followed by immunoprecipitation of the desired brain-derived exosomes using magnetic beads coupled to the appropriate antibody. The exosomes were lysed and α -synuclein was measured in each sample using electrochemiluminescence ELISA (Meso Scale Discovery).

Results: α -Synuclein concentration levels separated the PD and control groups with moderate, sensitivity and specificity, whereas the MSA and control groups were separated with high sensitivity and specificity. The ratio between the α -synuclein concentration in oligodendroglial and neuronal exosomes distinguished between the PD and MSA groups with high sensitivity and specificity. This ratio also correlated with disease progression in the PD cohort. In postmortem samples, substantial erythrocyte hemolysis caused contamination of the samples by erythrocytic α -synuclein, preventing the use of these samples for validation of diagnosis in our assay.

Conclusions: α -Synuclein in brain-derived exosomes potentially is a useful blood biomarker for differential diagnosis of PD and MSA. In particular, the ratio between the α -synuclein concentration in oligodendroglial and neuronal exosomes separates PD from MSA with high sensitivity and specificity and may also be used as a progression biomarker. Postmortem samples cannot be used in this particular assay due to hemolysis, but may be useful for other biomarkers that are not affected by hemolysis.

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Title (Please, note the Title is changed)

Are the bio-molecular traits of fibrils grown in vitro related to the fibrils formed in disease?

First and Last Name of Authors

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Abstract:

Background: Aggregated α -synuclein (α -syn) accumulates as cytoplasmic inclusions in many neurodegenerative diseases, collectively known as synucleinopathies. Multiple system atrophy (MSA) is a rare synuclein-related neurodegenerative disorder that currently lacks a disease-modifying therapy. In glial cells of MSA brains, aggregated α -syn accumulates as glial cytoplasmic inclusions (GCI). α -Syn is also the main constituent of Lewy bodies (LB), which are found in Parkinson's disease (PD) and dementia with Lewy bodies (DLB). Evidence suggests that the pathological accumulations of α -syn are fibrillar assemblies, known as amyloid fibrils, and the toxicity of these species is structure specific.

Aims/Purpose: Depending on the assembly conditions, α -syn may form fibrils with variable structures. This structural polymorphism of α -syn fibrils raises the question of whether fibrils grown *in vitro* faithfully represent fibrils formed in the brain tissues with α -syn inclusions. To answer this question, we examined α -syn fibrils formed in the presence of brain tissues with GCI. For these studies, we were governed by two common assumptions about amyloid formation: (1) mature fibrils can accurately template their conformation on to the other proteins, and (2) the templating process persists in the sequentially repeated reactions.

Results and Discussion: Our results suggest that α -syn fibrils grown in the presence of GCI-containing tissue differ than fibrils grown of recombinant protein only. The GCI-seeded fibrils have specific reactivity to conformational antibodies, and display distinct morphology when examined with electron microscopy. Furthermore, we found that the tissue-seeded fibril formation deviates from the classical amyloid formation and does not strictly follow the template-driven mechanism of cyclic amplification. Because of the association of α -syn accumulation with multiple diseases, developing strategies for precise extraction of *in-vivo*-grown fibrils is critically important. Thus, conducting more realistic studies of the fibrillation process is necessary in order to gain valuable insights in understanding synuclein-related disorders and potentially attain appropriate therapeutic agents.

Title: GLOMERULAR AND VASCULAR AMYLOIDOGENESIS: THE ROLE OF MESANGIAL CELLS AND PERICYTES IN AL-AMYLOIDOSIS.

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Aims: The kidney is a common organ affected in AL-amyloidosis, since about 50% of AL patients have renal involvement at the time of diagnosis^[1]. Renal amyloid deposition first occurs in the mesangium and in the renal vasculature and evidence indicates that both mesangial cells and pericytes/smooth muscle cells play a role in light chain amyloidogenesis^[2]. Mesangial cells and pericytes/smooth muscle cells transform into facultative macrophages to be able to perform functions needed to accomplish the process of amyloidogenesis. It has been previously shown that both cell types can selectively internalize amyloidogenic light chains^[3]. Once internalized, the light chain is routed to the lysosomal compartment, where its aggregation into amyloid fibril occurs. The amyloid is then extruded to the extracellular compartment^[4]. This study aimed to identify the membrane receptor that mediate the internalization of the amyloidogenic light chains in the mesangial and pericytes/smooth muscle cells, as well as the signaling events that regulate the trafficking of the amyloidogenic protein into the lysosomal compartment and the phenotype transition. Based on the experimental evidence, a seeding-mediated mechanism of spreading of the light chain amyloid deposition in the kidney is proposed.

Methods: The study was performed with several light chains purified from the urine of patients with renal biopsy-proven AL-amyloidosis. For comparison purpose, light chains obtained from patients with light chain deposition disease (LCDD) and myeloma cast nephropathy were also used. Three different experimental platforms [(in-vitro (cellular model), ex-vivo (organ model) and in-vivo (animal model))] were used to unravel the sequence of pathological triggered by the interaction of the light chains with the receptor in the membrane of the cells. Several techniques, which included light, electron (with and without immune-gold labeling) and scanning electron microscopy, immunofluorescence and immunohistochemistry, along with mass spectroscopy were used to delineate the process of cell-mediated AL-amyloid formation in the kidney.

Results and Discussion:

When mesangial cells are incubated with both amyloidogenic and LCDD light chains, they exhibit characteristic surface alterations with development of caveolae (coated pits) (Figure Panel A). Analysis with transmission (using immunogold techniques) and scanning electron microscopy indicates that light chains interact with the cell membrane through activation of SORL1 receptors and mediators located in these caveolae^[5]. Both, amyloidogenic and LCDD light chains compete with SORL1 receptor, but only the first type of light chain is internalized by the cell (Figure panels B, C, E). The interaction with the receptor results in the internalized protein to be routed to the mature lysosomal compartment, processed in the acidic lysosomal environment, and the generation of amyloid fibrils which are then extruded to the extracellular compartment (Insert panel A). This process is modified according to the physicochemical characteristics of the involved light chains and the external milieu affecting speed of the fibril formation. Once the process of amyloidogenesis begins, the deposited fibrils in the extracellular space, resulting from extrusion from mesangial cells^[4], create the nidus for further amyloid formation, eliminating the need for cellular elements. We propose that the ability of mesangial cells, as well as pericytes/smooth muscle cells, to promote the amyloid aggregation of the light chain internally, and then extrude the aggregate to the extracellular space contribute the amyloid deposition in the kidney (Figure panel F). The amyloid produced by these cells nucleates the subsequent aggregation of soluble precursor protein accelerating fibril formation. Amyloid seeding is a recognized potent mechanism of amplification and spread of disease-related fibrils completely independent of cells. We found that increasing lysosomal pH, and thrombospondin / transforming growth factor also decrease amyloid formation.

Conclusions:

Both mesangial cells and smooth muscle cells are key participants in renal amyloidogenesis. Their ability to transform phenotypically to a macrophage phenotype and internalize light chains is essential for forming amyloid fibrils. Signaling events and other steps can be pharmacologically modulated. Altering the milieu, externally and internally, modifies the amount and speed of amyloid formation. There are also a number of steps in the process of amyloid formation that may be pharmacologically modulated to avoid, decrease and or abolish amyloid formation.

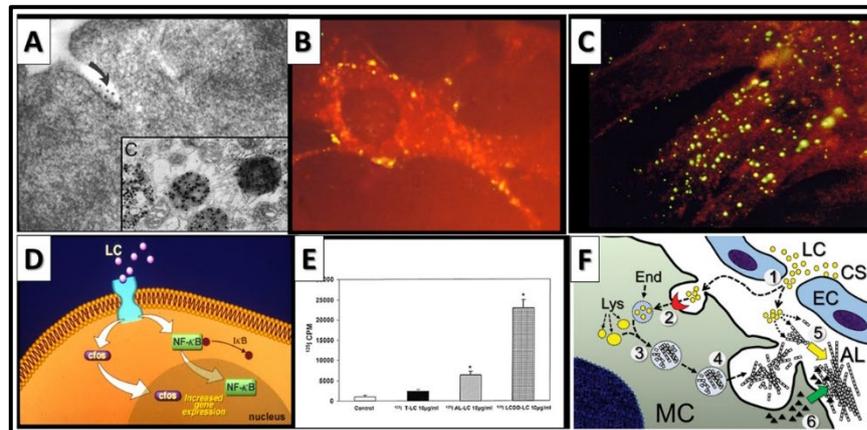


Figure. Mechanism of amyloidogenesis in MCs incubated with amyloidogenic LCs. **A)** Initial interaction of the light chain with surface caveolae in mesangial cells where receptor resides, after 30 seconds post incubation. The image shows TEM analysis with immunogold labeling (10 nm gold particles) for λ light chain in mesangial cells incubated with an amyloidogenic λ light chain. Insert: Same light chain after 5 hours post incubation in mature lysosomes. Magnif. A: x35,000, insert: x17,500. **B)** Direct fluorescence of mesangial cells coincubated with Texas red-labeled (red) amyloidogenic light chain and fluorescein (green)-labeled LCDD light chain. Both light chain types compete for the same receptor on the surface of mesangial cells (co-localization showing yellow staining). Note that amyloidogenic light chains are avidly internalized. Magnif: x500. **C)** Internalization of fluorescein isothiocyanate-labelled amyloidogenic light chains into mesangial cells detected by direct fluorescence. Magnif. x500. **D)** Schematic representation of interactions of glomerulopathic (amyloidogenic and LCDD) light chains with mesangial cells and activation of c-fos and NF- κ B to activate downward cellular pathways. **E)** Comparison of various types of 125 I-labelled light chains binding to mesangial cells at 30 minutes of incubation. Note prominent interaction of LCDD light chains with surface of mesangial cells and lesser but significant of amyloidogenic light chains. The amyloidogenic light chains are avidly internalized. Images shown in panels B, D, E, and G were taken from previously published material^[3]. **F)** Diagrammatic representation of AL amyloid formation by mesangial cells. **1)** The unstable and misfolding-prone monoclonal light chain (yellow circles) is filtered from the glomerular capillary. **2)** The LC internalizes into the MCs by a receptor-mediated mechanism. **3)** The early endosome (End) containing the misfolded LC fuses with the lysosomes (Lys), transforming into a late lysosome, where the self-assembly of the LC into amyloid fibrils occur. **4)** The fibrils formed inside the lysosomes are extruded from the MC, accumulating in the extracellular space. **5)** Soluble monomers of the monoclonal LCs aggregate into the preformed fibrils, which seed the aggregation reaction. Fibrils accumulate in the extracellular space. **6)** MMPs and other proteases secreted by the mesangial cells proteolyze the light chain amyloid, removing the protease-sensitive light chain constant domain, as well as other components of the extracellular matrix^[6].

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Can Fluorescent Probes Aid in Ante-mortem Diagnosis of Amyloid-Associated Diseases?

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Abstract:

Aims

The goal of this research is to develop new fluorescent probes that can detect amyloid deposits in tissue¹ and amyloid-like aggregates in biological fluid samples² as a novel approach to ante-mortem detection of amyloid-related disease. This presentation will include some recent results that show we can detect amyloid deposits in the brains and retinas of transgenic Alzheimer's disease (AD) and prion (PrP)-inoculated mice, and in human cadaver samples.

Methods

Transgenic APP/PS1 mice or prion-inoculated mice were treated with a fluorescent, amyloid-targeting molecule via IP injection and subjected to ante-mortem fluorescence retinal imaging. Both the brain and retinal tissue from these mice were also inspected for amyloid deposits post-mortem using immunohistochemistry. Additionally, eyes and brains collected post-mortem from human cadavers of AD or control subjects were stained with the fluorescent probe or antibodies for Aβ and inspected for the presence of amyloid deposits.

Results

Small, fluorescently stained puncta were observed in the retinas of APP/PS1 mice treated with the fluorescent amyloid-targeting probe, while large continuous areas of bright fluorescence were observed permeating radially from the optic nerve in prion-inoculated mice treated with the probe. Neither of these types of labelled deposits were present in the retinas of wild-type mice under the same treatment conditions. Staining of human retinas from AD patients revealed fluorescent puncta that resembled the puncta found in the retinas of APP/PS1 mice, and these puncta were more prevalent in AD patients than in control patients.

Conclusion

A fluorescent amyloid-targeting probe enables ante-mortem detection of Aβ or PrP-containing puncta in the retinas of mouse models for AD or prion disease and post-mortem detection of puncta in the retinas of human AD patients, suggesting this probe may be useful for detection of retinal biomarkers in living AD patients.

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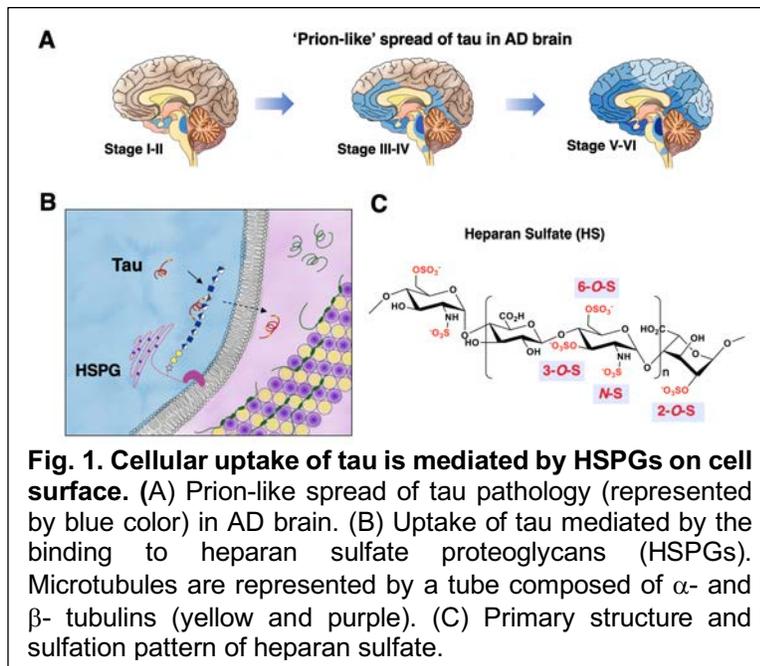
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²J.P. Do, K.J. Cao, S. Wei, L.C. Laurent, M.M. Parast, and J. Yang *Anal. Chem.*, **2018**, *90*, 14316-14320

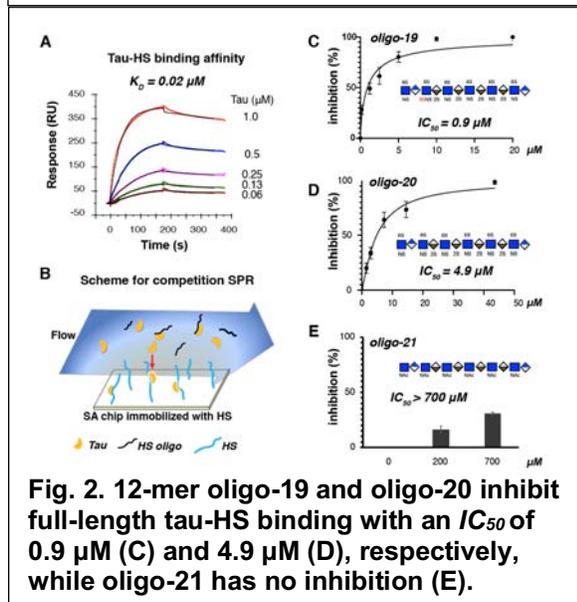
Rare 3-O-sulfation of Heparan Sulfate Enhances Tau Interaction, Cellular Uptake and Seeding

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Aims (Fig. 1). In normal brain physiology, the microtubule-associated protein tau binds and stabilizes microtubules in neurons. In Alzheimer's disease (AD), tau dissociates from microtubules and aggregates to form neurofibrillary tangles (NFT), a pathological hallmark of AD. The extent of NFT pathology correlates well with cognitive decline in AD¹ and is used for staging the disease. Recently, mounting evidence establishes that tau pathology spreads through neural networks by transcellular movement in an orderly and prion-like manner²⁻⁴. Heparan sulfate proteoglycans (HSPGs) are ubiquitously present on neuronal surface and have been shown to bind tau, facilitating tau cellular uptake and seeding of tau aggregation in the recipient cell⁵⁻⁸. HSPGs are composed of HS chains



covalently linked to a protein core. Interfering with tau-HS interaction using HS mimetics can inhibit tau transcellular migration in cell culture and animal models^{5,9,10}. Specific sulfation patterns are important for glycan/protein interactions^{11,12}. The repeating disaccharide unit of HS is a glucuronic acid (GlcA), and to a lesser extent an iduronic acid (IdoA), linked to an N-acetylglucosamine (GlcNAc). HS can be sulfated at the 3-OH, 6-OH and/or NH of the glucosamine residue, and the 2-OH of the uronic acid residue. We have previously identified 6-O-sulfo group (6S) as an important determinant in tau-glycan interaction¹³, which was later confirmed by others with a variety of approaches^{7,8}. *In this study, we characterized the role of the rare 3-O-sulfation (3S) in tau-HS interaction, cellular uptake and tau seeding.*

Methods. We used glycan array, SPR, NMR titration, cell surface binding and uptake assays with Alex488-labeled tau with HS mutant cell lines, and tau seeding experiments using the biosensor cell line.

Results. We demonstrate that the 3-O-sulfation strongly enhances the tau-HS interaction and cellular uptake of tau, using LMHS microarray, SPR, cellular binding (Fig. 2) and uptake assays, and NMR (Fig. 3). An HS 12-mer with one additional 3-O-S (oligo-19) inhibits tau-HS interaction with ~5-fold lower IC_{50} value than the same HS 12-mer without 3-O-S (oligo-20) (Fig. 1). The reduced cell surface binding and internalization of tau in *Hs3st1*^{-/-} cells indicates that 3-O-sulfation significantly enhances the cellular uptake of tau (data not shown here).

NMR mapping shows 3-O-S (Fig. 2) preferably binds to the PRR2 and R2 domain of full-length tau, which are the crucial regions for aggregation, MTs association, and interaction with heparin and other proteins. We also studied inhibition of tau seeding by 3S-containing oligosaccharide (Fig 4), by comparing the inhibition of tau seeding by oligos-19 and 20 from the glycan array. Oligo-19 and -20 have the exact same composition except for a 3S group on oligo-19. Tau aggregates extracted from htau mouse model was here used as the aggregation seed added to cell culture medium.

In Fig. 13B, at 5 μ M oligo, compound 19 clearly reduces intracellular tau aggregation observed by fluorescence microscopy and FRET. The enhanced inhibition by 3S is even more pronounced at 15 μ M oligo concentration.

Conclusion. Our results demonstrate the key role of 3-O-S in the tau-HS interaction, cellular uptake of tau, intracellular seeding, uncovering a unique structural requirement of HS recognition by tau. This work represents a major step forward in our understanding of the mechanism of tau-HS interaction¹⁴, with important implications for 3-O-S as a pharmacophore targeting the spread of tau pathology in the development of effective AD therapy.

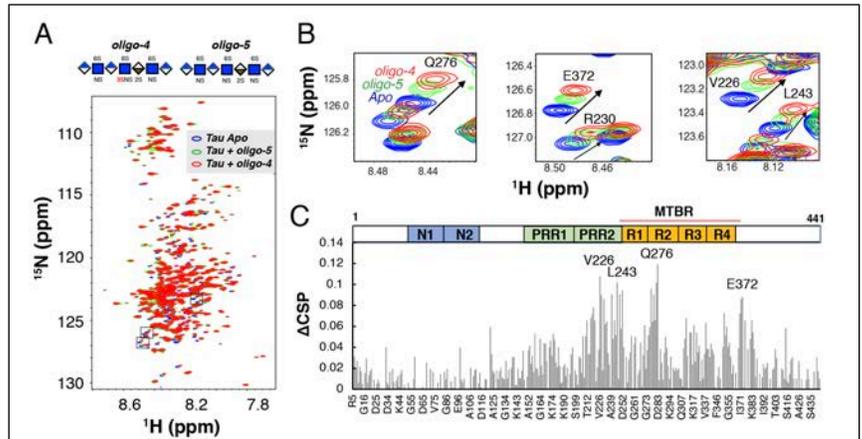


Fig. 3. Chemical shift perturbation difference (Δ CSP) reveals interactions between 3-O-S and PRR2 and R2 domain of full-length tau.

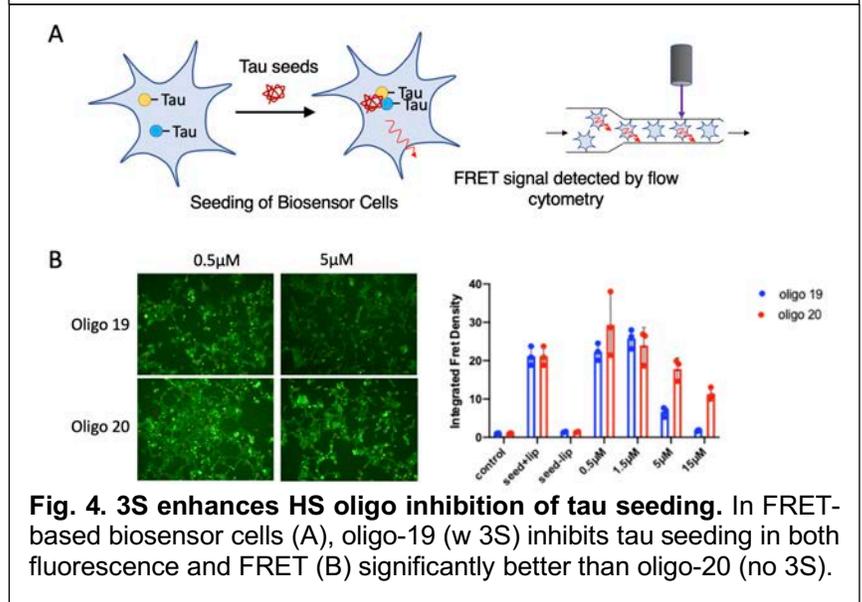


Fig. 4. 3S enhances HS oligo inhibition of tau seeding. In FRET-based biosensor cells (A), oligo-19 (w 3S) inhibits tau seeding in both fluorescence and FRET (B) significantly better than oligo-20 (no 3S).

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Structural Basis for Action of Serum Amyloid A in Lipid Transport and Acute-Phase Response

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Aims: Serum amyloid A (SAA, 12 kDa) is an enigmatic biomarker of inflammation best known as a protein precursor of AA amyloidosis, a major complication of chronic inflammation. Beneficial functions of this Cambrian protein remain obscure. SAA has been highly evolutionally conserved for at least 500 million years, suggesting that this protein family played a vital role throughout evolution. In acute inflammation, infection or after injury, plasma levels of inducible SAA increase rapidly and dramatically up to ~1,000 fold, reaching up to 3 mg/ml, and then swiftly drop, suggesting that SAA plays an important role in the acute-phase response. Our goal is to determine this role.

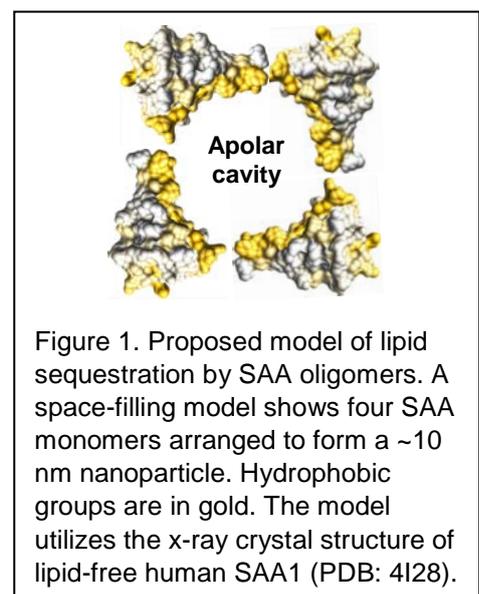
We noted that during inflammation SAA increases simultaneously with secretory phospholipase A₂ (sPLA₂), another ancient lipophilic acute-phase reactant. Both proteins increase systemically in plasma, as well as locally at the inflammation sites. This spatiotemporal overlap compelled us to determine whether SAA influences the phospholipid hydrolysis by sPLA₂.

Approach: We used full-length recombinant murine SAA1 (also known as SAA2 based on its gene sequence) produced in the laboratory of Dr. Marcus Fandrich in Ulm, Germany. We tested whether the protein could spontaneously solubilize diverse lipids and their degradation products, and used an array of biochemical and biophysical techniques to characterize these products and to determine whether and how SAA influences enzymatic activity of sPLA₂.

Results and Discussion: Our *in vitro* studies showed that SAA solubilized bilayers of diverse phospholipids to form lipoprotein nanoparticles that provided excellent substrates for sPLA₂, which does not act on flat lipid bilayers. Moreover, SAA sequestered free fatty acids and lysophospholipids, which are the hydrolytic products of sPLA₂, to form stable proteolysis-resistant complexes. Thereby, SAA promoted the sPLA₂ activity via a dual effect: i) it solubilized lipid bilayers to generate substrates for sPLA₂, and ii) it removed the bioactive water-insoluble products, which is necessary for the reaction to proceed. Importantly, unlike albumin, SAA effectively sequestered free fatty acids under acidic conditions, which characterize inflammation sites. This suggests that SAA can effectively substitute for albumin at the inflammation sites to remove the products of sPLA₂ and other lipases. Similarly, SAA can remove the products of lipid oxidation. This ability to solubilize a wide range of lipids and their degradation products suggests that SAA acts as a lipid scavenger *in vivo*, which we propose is key to its vital housekeeping role in clearing cell membrane debris.

Conclusions: SAA and sPLA₂ can act in synergy to remove cellular membrane debris from the sites of injury, which is a prerequisite for tissue healing. We postulate that such a removal of diverse lipids and their degradation products constitutes a vital evolutionally conserved role of SAA in acute-phase response and innate immunity.

Our ongoing experimental and computational studies support this idea and provide new insights into the structural underpinnings for SAA-lipid interactions and formation of SAA-lipid nanoparticles (Figure 1). These studies indicate that lipids are sequestered in the hydrophobic cavity formed by the apolar faces of helices h1 and h3 (gold surface in Figure 1) to form heterogeneous oligomeric complexes. This binding mode explains the lipid binding promiscuity of SAA and distinguishes it from albumin or any other lipophilic proteins. The details of the SAA solution conformations in lipid-free and lipid-bound forms and how they are converted into a cross- β -sheet in amyloid are being elucidated.



How does A β self-aggregate on and exert toxicity against neuronal cells?

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Abstract:

Purpose: Accumulating evidence suggests that membranes play a pivotal role in the self-assembly of A β . We have investigated A β -lipid bilayer interactions, and found that A β specifically binds to a cluster of GM1 ganglioside, changing its conformation from an α -helix rich one to an β -sheet rich one, eventually forming amyloids [1]. The amyloids are cytotoxic and possess a unique, tape-like structure composed of a single layer of mixed in-register parallel and 2-residue-shifted antiparallel β -sheets [2]. The purpose of this study was to elucidate detailed mechanisms by which A β self-aggregates on and exerts toxicity against neuronal cells using various physicochemical and cell biological methods.

Methods: Aggregation of A β doped with HL647-A β on neuronal SH-SY5Y cells was monitored by fluorescence correlation spectroscopy (FCS) and amyloid formation was detected with Congo red. The activation of caspases was detected by immunoblotting and immunofluorescence. Intracellular localization of NF κ B was visualized with RelA-GFP. ROS and Ca²⁺ were detected with dichlorofluorescein and Fluo 4, respectively.

Results: FCS experiments revealed that A β formed oligomers (~15mer) before amyloid formation. Immunofluorescence experiments suggested that these oligomers were nontoxic, whereas amyloids induced apoptosis [3]. In contrast, amyloids formed in aqueous solution did not trigger apoptosis. We confirmed this also by immunoblotting. Inhibitor experiments suggested that Toll-like receptor (TLR) 4/TLR 6-NF κ B signaling pathway and Nod-like receptor family, pyrin domain-containing (NLPP) 3 inflammasomes were activated. Upon amyloid formation, NF κ B was translocated into the nucleus. An increase in intracellular Ca²⁺ concentration as well as the generation of reactive oxygen species (ROS) were also observed, suggesting mitochondrial damages. These did not occur when A β was in the oligomeric state. Knockout cell studies indicated that apoptosis was induced via a caspase 8 --> caspase 9 --> caspase 3 route.

Conclusion: In contrast to aqueous phase aggregation, amyloid fibrils are the culprit for apoptosis, in which inflammation pathways play an important role. The key molecules that initiate the inflammation responses, such as TLRs and NLRP3, are promising targets to reduce apoptotic cell death induced by A β .

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Innovating high-resolution novel imaging approaches to elucidate mechanisms of prion-like spreading of neurodegenerative disease

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Stanford University

Abstract: An exciting new area in neurodegenerative disease research is the emerging phenomenon of prion-like spreading of neurodegenerative disease proteins. Many fundamental questions and challenges lay ahead. 1) What is the mechanism of trans-neuronal spread of pathology? Is the transport based on diffusion? Is it trans-synaptic? If trans-synaptic, is it anterograde or retrograde? If we can define these, then we can have some footholds to devise strategies aimed at blocking, or at least slowing down spread. 2) Parkinson's Disease (PD) is associated with a bafflingly diverse array of genetic susceptibility factors. Do some or all of these converge on misfolding and spread of protein aggregates? Any therapeutic or mechanistic investigation into prion-like spreading will require the development of powerful new imaging approaches in living animals. Simply put, we need a way to monitor the impact of these aggregates on brain activity as they spread, in a living animal, from one neuron to the next, tracking their paths, figuring out why they take some routes but not others and how this spread affects and is affected by neuronal activity. We are starting to tackle these challenges in order to provide fundamental mechanistic insight into this fascinating new role of prion-like spread of neurodegenerative disease.

Methods/Results: Previous reports have demonstrated that local delivery of α -syn pre-formed fibrils (PFFs) into the striatum of rodents induces aggregation of endogenously expressed α -syn in remote brain areas projecting to the injection site, including the *substantia nigra pars compacta* (SNpc) (Luk, Kehm et al. 2012, Peelaerts, Bousset et al. 2015). PFF-based in vivo mouse models allow for both the ability to perform longitudinal studies, as well as to assess diverse neuronal populations and the complex interplay between neurons and glial cells in the context of neurodegeneration (Luna, Decker et al. 2018, Yun, Kam et al. 2018). We established this model in our laboratories at Stanford and recently harnessed this model to test the impact of mutations in the *LRRK2* gene on α -syn aggregation and degeneration (Bieri, Brahic et al. 2019).

We unilaterally injected wild-type mice with sonicated pre-formed α -syn fibrils in the dorsal striatum and sacrificed animals at specific time points (2 weeks, 2, 4, 8, 12, 18 months post injection). We then processed samples for iDISCO brain clearing and immunostaining to detect phosphorylated α -syn (S129). We wrote custom scripts in Python and R to down-sample the autofluorescence data for a single brain from the acquired resolution (4.0625 μ m lateral, 3 μ m depth) to an isotropic 25 μ m resolution and then registered data to the Allen Institute 25 μ m average anatomical template atlas. This was performed automatically using a combination of affine and b-spline transformation registrations. The resulting pair of transformations is used in subsequent steps to transform coordinates in the raw data space to the template atlas space. The secondary antibody fluorescence data was used to determine the locations of large aggregates of α -syn [phosphor S129]. Background fluorescence from this volumetric data was first removed by subtraction of the morphological opening of the original image, with a structure element size that was experimentally determined to be 5 pixels in each spatial dimension. The local maxima of the resulting image are obtained, and all of these points are used as the starting points for a watershed segmentation, with a threshold

background intensity parameter that was experimentally determined. The radius of each aggregate is then calculated from this binarized watershed image. Points are filtered using a combination of their intensity value and radius in order to exclude aggregates that are too small to accurately consider, or too large to actually be contained within an individual neuron. After down-sampling each aggregate point to the 25 μm resolution and applying the optimized registration transformation, the numbers of aggregates were counted at each voxel in this atlas space, with the resulting volume being called a "heatmap." Voxel-level statistics across brains acquired at two different time points involved first low pass filtering each heatmap and running a two-sided t-test at each voxel across the two groups. The transformed locations of each α -syn [phosphor S129] aggregate were also further grouped into the 700 different anatomically segmented regions in the Allen Atlas for further statistical analysis between longitudinal groups. Computational model assuming symmetry of the two hemispheres, time invariance, and additively was constructed. The ideal model would be able to predict the future pathological state given a current state: $Wx_t = x_{t+1}$. Error was quantified by L2 norm of difference between the actual next state (x_{t+1}) and the predicted next state (Wx_t). Preliminary analysis show that the spreading pattern is clearly not a simple diffusion pattern and that it is likely retrograde.

Discussion and/or Conclusion: α -syn fibril was injected into striatum and α -syn pathology spread was imaged using iDISCO at different time points for 18 months. Quantification across the brain using 3D imaging and computational methods reveal spreading that follow synaptic pathways.

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Golgi defects in Alzheimer's disease

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Abstract:

Introduction: Golgi fragmentation occurs in neurons of patients with Alzheimer's disease (AD), but the underlying molecular mechanism causing the defects and the subsequent effects on disease development remain largely unknown. At the last conference, we reported the confirmation of Golgi fragmentation in human AD patient brain tissues and APP^{swe}/PS1 Δ E9 transgenic mouse and tissue culture models. Subsequently, we have determined the underlying mechanism and functional consequences of Golgi fragmentation in AD.

Results: In the AD mouse model, Golgi defects are present prior to A β deposition and does not correlate with the proximity to plaques. In tissue culture, oligomeric A β exhibits stronger effects to induce Golgi fragmentation and toxicity in neurons than A β monomers and fibers [1]. Our results suggest that A β accumulation leads to Golgi fragmentation by activating cdk5, which in turn phosphorylates GRASP65 and perhaps other key proteins critical for maintaining Golgi morphology. Significantly, rescue of Golgi structural defects by inhibiting cdk5 or by expressing nonphosphorylatable mutants of GRASP65 reduces A β secretion by elevating non-amyloidogenic APP cleavage. Significantly, we provide experimental evidence that A β -induced Golgi defects precede and correlate with neurotoxicity *in vitro* and that rescue of Golgi structure reduces A β toxicity [2]. Finally, we show that stabilizing the structure of the Golgi by expressing a non-phosphorylatable mutant of the Golgi stacking protein GRASP65 attenuates A β -induced neurotoxicity *in vitro* and *in vivo*.

Discussion: These results reveal Golgi fragmentation as an important mechanism through which A β may exert its toxic effects. A major potential unrecognized source of A β toxicity may be that it compromises Golgi integrity and perturbs the proper trafficking and processing of many proteins essential for neuronal function. Our results further implicate Golgi structural abnormalities in the pathogenesis of AD and highlight a functional role for Golgi stacking defects in neurodegeneration. Our study provides a molecular mechanism for Golgi fragmentation and its effects on APP trafficking and processing in AD, suggesting Golgi as a potential drug target for AD treatment [3-5]. We are applying a similar approach to determine Golgi defects in other diseases such as Inclusion body myopathy with early-onset Paget disease and frontotemporal dementia (IBMPFD) [6], asthma, and cancer [7].

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Potential Role of Amyloid Oligomer Interactions in Tau Oligomers Polymorphisms

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Neurodegenerative diseases are complex diseases characterized by one or more disease-specific protein aggregates and clinical symptoms. However, considerable overlapping has been observed between multiple neurodegenerative diseases, both in terms of protein accumulation and clinical manifestations. Tau aggregation is a common factor in many different neurodegenerative diseases, other proteins such as α -synuclein, TDP43 are also found in different diseases of subgroup of patients.

Recent studies of amyloid polymorphisms (strains) and the toxic interactions between amyloidogenic proteins represent important first steps to elucidate the toxic interplay between soluble aggregates and should be studied in vitro using multiple approaches and in vivo at different disease stages.

Our results suggest that occurrence of soluble aggregates of α -synuclein, Amyloid- β , TDP43 and others, not only synergize with tau and aggravate its toxicity, but also play a role in tau soluble aggregates polymorphisms and the formation of oligomeric strains.

Understanding the role of different polymorphic structures is critical groundwork for the development of multi-panel biomarkers and perhaps more successful therapeutic interventions by targeting multiple candidate and personalized therapeutics.

The lipid-chaperone hypothesis: May amylin, α -synuclein and A β have a common molecular mechanism for membrane damage?

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Purpose: Many researchers over the past thirty years have described a class of polypeptides, including A β peptide, α -synuclein, and IAPP, that form pathogenic amyloid aggregates in tissues of patients who have Alzheimer's, Parkinson's, and type 2 diabetes mellitus diseases, respectively. These proteins, also called intrinsically disordered proteins (IDPs), have a highly dynamic conformational ensemble. Upon interacting with other cytosolic partners such as proteins or lipid membranes, IDPs can partly (mis)fold into (dys)functional conformations and accumulate as amyloid aggregates. The toxicity of amyloid proteins is often linked to membrane damage associated mechanisms¹⁻³. However, the molecular details of the membrane disruption processes are still unknown. Current hypotheses include: (i) generation of stable transmembrane protein pores (toxic oligomer hypothesis); (ii) membrane destabilization via a "carpet model"; (iii) lipid extraction by amyloids via a "detergent-like" mechanism and (iv) membrane damage by lipid peroxidation (inflammation/oxidative stress hypothesis). It is unclear whether these models are mutually exclusive or if (and how) they cooperate to trigger membrane damage. The majority of literature focuses on lipid-protein interactions occurring at the bilayer, rather than interactions in the aqueous phase. However, a chemical equilibrium exists between monodispersed lipids and their self-assemblies, described by a critical micellar concentration (CMC). In some cases, (e.g. for molecules with short lipid tails or negatively charged), the concentration of free lipids in the aqueous phase may reach values in the μ M range. Since proteins are also present at μ M concentrations in most of the amyloid/membrane interactions assays, it is plausible that a lipid-protein binding equilibrium exists in the aqueous phase, which might influence their membrane insertion. Recently, a phenomenological model⁴ based on experimental findings was proposed to simulate the transfer kinetics of a lipid-protein complex from water to the lipid-bilayer phase. We find that the water-soluble lipid-protein complex inserts into the membrane faster than the free protein due to the hydrophobic differences between the lipid-protein complex and the bare protein. This model is supported by several experiments carried out on human IAPP⁵. Importantly, our results offer a novel mechanistic explanation as to why bilayer thickness is inversely correlated to membrane damage induced by amyloid proteins. The increased bilayer damage seen for thin bilayers is caused by an increase of lipid-bound proteins causing faster insertion and membrane disruption as there are more free lipids in solution for the shorter acyl-chains (high CMC). Thus, it is not the thickness of the bilayer that is important in causing membrane damage, but rather the relative free lipid concentration in solution, coupled to the stability of the lipid-protein complex and its hydrophobicity. This work provides both computational and experimental evidence pointing a critical role played by free lipids in driving membrane poration mechanisms and fibril formation.

Methods: We tested our hypothesis on different amyloidogenic proteins, including A β peptide and α -synuclein. Further, we also investigated two non-amyloidogenic proteins, rat-IAPP and β -synuclein, as controls. Within this framework, we carried out membrane leakage experiments using fluorescence and vesicles composed of different lipid chain lengths with CMC ranging from nM to μ M. Molecular dynamics simulations were employed to evaluate the stability of lipid-protein complexes in water. Finally, the formation of water-soluble lipid/peptide complexes was characterized by circular dichroism spectroscopy and 2D NMR measurements.

Conclusion: Here, we propose a general mechanism of lipid-assisted amyloid penetration into the membrane able to explain toxicity/membrane damage of IDPs: The lipid-chaperone hypothesis. Our biophysical investigations suggest that free lipid plays a pivotal role in membrane damage, and the

same molecular mechanism is shared among IAPP, A β , and α -synuclein. Our results suggest that it is important to consider the many roles of membrane in the development of amyloid inhibitors and potential compounds to treat these devastating amyloid diseases.

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ELUCIDATING THE MECHANISM OF IMMUNOGLOBULIN LIGHT CHAIN AMYLOID AGGREGATION

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Abstract:

Aims

Under certain circumstances, the immunoglobulin light chains (LCs) can lose the native folding and deposit in the extracellular compartment in the form of insoluble fibrillar aggregates, causing AL amyloidosis (1). Since not all monoclonal LCs are amyloidogenic, there has been much interest in elucidating what confers the LC the ability to form amyloid. Recent studies revealed, for the first time, the internal structure of the AL fibrils (2-4). However, the understanding of how the LC reaches the fibrillar state remains quite incomplete. This is in part due to the methodological challenge that the structural characterization of the non-native intermediates implies. To meet that challenge, we generated conformation-sensitive antibodies that recognize the amyloid-like fibrils of 6aJL2 protein, as well as non-native species that populate its fibrillogenesis pathway. We combined immunoassays with these antibodies and an array of computational, spectroscopic and biochemical methods to identify pro-fibrillogenic segments of 6aJL2 protein and characterize the structural changes accompany its aggregation into amyloid-like fibrils.

Methods

Protein 6aJL2 is a recombinant (r) λ 6 variable domain (V_L) encoded by the germline of the rV_L gene segment *IGLV6-57* (6a) (5). This gene segment is strongly associated with amyloidosis (6). Five different conformation-sensitive rabbit polyclonal antibodies were generated by immunizing the animals with synthetic peptides with the sequence of specific regions of 6aJL2 protein (Figure 1A). The specificity of these antibodies was determined in dot-blot assays and transmission electron microscopy-immunogold labeling analysis (TEM-IGL). These methods were also applied to characterize the non-native species populating the aggregation pathway of 6aJL2 protein (Figure 1B-D).

The fibrillogenic hotspots of 6aJL2 protein were identified combining two complementary approaches (7). One of them was based on several web-based computational tools optimized to predict fibrillogenic/aggregation-prone sequences. Then, the predictions were confirmed with an ad-hoc synthetic peptide library. In the second approach, 6aJL2 protein was proteolyzed with trypsin, and the products incubated in aggregation-promoting conditions. Then, the aggregation-prone fragments were identified by combining standard proteomic methods, and the results validated with a set of synthetic peptides with the sequence of the tryptic fragments. Residues playing a key role in the mechanism of aggregation of 6aJL2 protein were identified by site-directed mutagenesis.

Results and Discussion:

We identified three fragments of the 6aJL2 protein that form amyloid-like fibrils autonomously (7). One of them (Ser26-Arg39) spans the CDR1 and the β strand C and was proven to be the most fibrillogenic of all fragments. Proline- and alanine-scanning mutagenesis analyses identified residues key for aggregation of peptide Ser26-Arg39. Site-directed mutagenesis to Asp showed that several of the residues key for the aggregation of peptide Ser26-Arg39 are also important for the fibrillogenesis of the intact 6aJL2 protein. Immunoassays with conformation-sensitive antibodies suggest that the fibrillogenesis of 6aJL2 protein is accompanied by conformational rearrangements that affect the whole molecule (Figure 1C & D). The earliest rearrangements appear to target structural motifs with protective

function, which probably expose the fibrillogenic sequences at the β strand B and the CDR1 to intermolecular contacts that trigger the aggregation.

Conclusions: The $\lambda 6$ protein 6aJL2 contains several fibrillogenic sequences located at different regions of the molecule. The most fibrillogenic one is located at the CDR1 and the β strand C. The aggregation of the 6aJL2 protein into amyloid-like fibrils involves a structural rearrangement that affects most part of the molecule, which is in agreement with what is now known about the structure of the AL fibril of the $\lambda 6$ LCs (3, 4). The aggregation appears to be triggered by local structural adjustments that target protective structural motifs that subsequently progresses to a profound structural conversion of the protein.

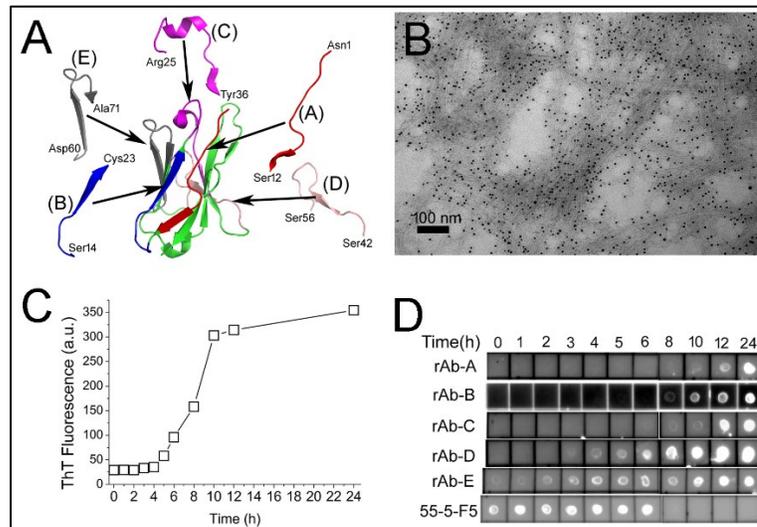


Figure 1. A) Segments of the V_L protein 6aJL2 used as immunogens for generating conformation-sensitive rabbit polyclonal antibodies that recognize the non-native forms of 6aJL2 protein (manuscript in preparation). The capital letters refer to the name given to the antibodies. **B)** Transmission electron microscopy analysis with immunogold-labeling of amyloid-like fibrils of 6aJL2 protein performed with the affinity-purified polyclonal rabbit antibody D (rAB-D). The secondary antibody was goat anti-rabbit IgG conjugated with 8 nm colloidal gold. **C)** *In vitro* fibrillogenesis of the V_L protein 6aJL2 determined by ThT fluorescence. **D)** Dot-blot assays for testing the ability of the five conformation-sensitive affinity-purified rabbit polyclonal antibodies to recognize non-native forms of 6aJL2 protein present in samples taken at different time of the fibrillogenesis shown in C). The antibody ID is shown on the left side of each panel. 55-5-F5 is a murine anti- $\lambda 6$ LC monoclonal antibody kindly given by Dr. Alan Solomon's group.

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Design and Early Insights of the AREPAS (A Role for Evaluation of PET Amyloid Status) Study

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Abstract: (Please use subsections such as Aims/Purpose, Methods, Results, Discussion and/or Conclusion)

BACKGROUND: Advances in biomarker detection have played a role in how we define Alzheimer's Disease (AD) in clinical research contexts. For example, the National Institute of Aging and Alzheimer's Association have published guidelines in 2018 suggesting amyloid PET imaging can play a role in defining AD in a clinical research paradigm. Furthermore, the IDEAS Study (Phase One) results revealed that amyloid PET scan can define a subpopulation of patients diagnosed clinically with AD but who are amyloid PET negative, as well as influence physician management. However, it is unclear if these nationwide-obtained results apply to clinical practice that is predominantly comprised of ethnic minorities. To investigate this, we developed an observational, longitudinal, and open-label cohort study: AREPAS (A Role for Evaluation of PET Amyloid Status).

SPECIFIC AIMS: To 1) evaluate the role of amyloid-beta PET in diagnosis of Alzheimer's Disease and 2) evaluate the role of amyloid-beta PET in clinical management of patients in a highly diverse Miami-based academic practice.

METHODS: We will seek to recruit 25 men and women of all ethnic groups aged 65 and older, with a diagnosis of dementia according to DSM-V and/or NIA-AA criteria, verified by a dementia specialist within 24 months, in whom the etiology of the cognitive impairment is uncertain but Alzheimer's Disease is a consideration for diagnostic etiology. It is expected that in these individuals, Amyloid beta PET status results would be expected to influence diagnosis and management.

RESULTS: As of October 31, 2019, the AREPAS study has recruited twelve participants. 67% of these patients identify as Hispanic, 25% as non-Hispanic white, 8% as non-Hispanic black. Of these, one withdrew consent prior to their scan, one is pending scan, and ten were scanned. Two of these individuals have tested negative on Amyloid PET imaging, and eight tested positive. They are pending follow-up visits. Data on changes in clinical management will be evaluated at the study's conclusion.

CONCLUSION: The AREPAS study hopes to complete enrollment and scans on or before May of 2020. Early insights of the study include a high proportion of Hispanic patients, and a low proportion (20%) of patients with clinical presentation suggestive of AD but with negative amyloid PET scans.

SUPPORT: This study is supported by a private research fund provided by Dr. Reza Khatib, as part of the KHATIB (Key Hallmarks of Amyloid Tracer as an Ideal Biomarker) project.

Suppression of Alpha Synuclein Membrane Toxicity by an Extracellular Chaperone

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Abstract:

Purpose

The aggregation of alpha synuclein (α S) into Lewy Bodies (LBs) is a clinical hallmark of Parkinson's disease (PD)¹. While LBs observed in PD are intracellular, emerging evidence suggests that extracellular α S also exists and contributes to PD pathology via a prion-like cell-to-cell transmission mechanism^{2,3}. Indeed, α S is secreted from cells via an unconventional exocytosis pathway that is independent of the endoplasmic reticulum and Golgi apparatus⁴. The extracellular α S is able to subsequently enter into recipient cells, with α S aggregates exhibiting enhanced propensity to internalize compared to monomeric forms^{5,6}.

While a substantial body of evidence now points to the presence of extracellular α S species, the specific interactions of such species with the extracellular proteome and its role in regulating α S internalization into cells is poorly understood. The extracellular milieu thus remains an untapped potential that could be harnessed for therapeutic interventions in PD. As a first step towards tapping the translational potential of the extracellular milieu, here we focus on the most abundantly expressed protein in blood plasma and CSF, *i.e.* Human Serum Albumin (HSA).

Methods

We have examined the interactions of defatted (rHSA) and non-defatted, endogenous (gHSA) HSA extracted from blood plasma with both monomeric and oligomeric α S. Subsequently, we evaluated how such interactions influence the association of α S with membranes. Our interrogation of the α S – HSA complexes employs a multi-disciplinary approach based on solution NMR and fluorescence spectroscopies, Dynamic Light Scattering (DLS), Size-Exclusion Chromatography with Multi-Angle LS (SEC-MALS), Transmission Electron Microscopy (TEM), Biolayer Interferometry (BLI) and Wide-angle X-ray Diffraction (WAXD).

Results

Our findings suggest that HSA inhibits α S oligomer (α S_n) toxicity through a three-pronged mechanism. First, endogenous HSA targets α S_n with sub μ M affinity via solvent exposed hydrophobic sites, breaking the catalytic cycle that promotes α S self-association. Second, HSA remodels low molecular weight (MW) oligomers and high MW fibrils into amorphous intermediates with reduced toxicity. Surprisingly, HSA also suppresses membrane interactions with the N-terminal and central α S regions.

Discussion

Overall, our comparative analyses of the α S – HSA interactions at progressive degrees of resolution uncover an unprecedented mechanism by which a model extracellular chaperone inhibits the toxicity of PD-associated α S oligomers and is summarized in Figure 1 below. On the same grounds, our results point to the notion that the extracellular proteostasis network plays a critical role in regulating the cell-to-cell transmission of α S not only by reducing the populations of membrane-binding competent α S oligomers, but also by shielding residual toxic species from the membrane interface. Our work thus underscores the importance of evaluating how these control mechanisms are dysregulated in diseased states. Unexpectedly, the data obtained here also lend support to the idea that chaperones not only assist in the folding and assembly of a protein into non-toxic species but may also prevent the interactions of toxic oligomers with membranes. The latter are known to promote the formation of toxic intermediates

and enhance neuronal dysfunction⁷. Lastly, the results presented here illustrate the effectiveness of our integrated experimental strategy to comprehensively probe at multiple length-scales protein-protein interactions involving a heterogeneous and transient amyloidogenic system.

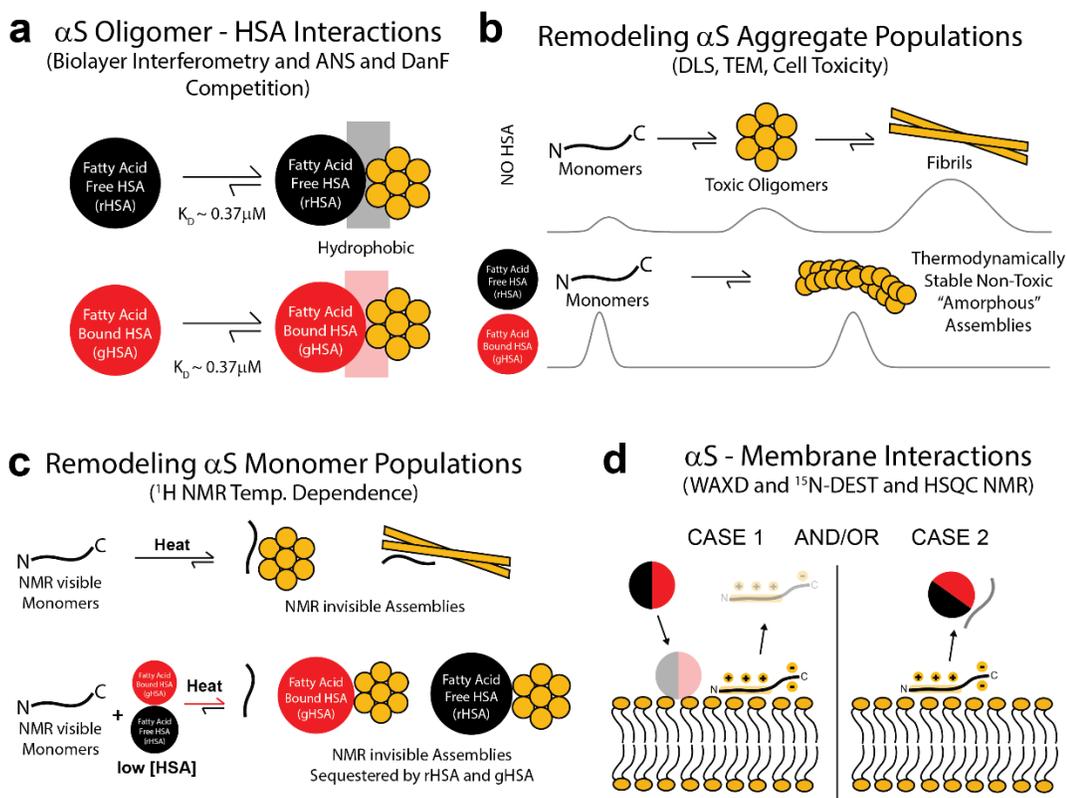


Figure 1 – Proposed mechanism for the inhibition of α S self-association and toxicity by human serum albumin.

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Granulins modulate liquid-liquid phase separation and aggregation of TDP-43 C-terminal domain.

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Purpose: Cysteine rich granulins (GRNs) have been shown to augment the toxicity of TAR DNA binding protein-43 (TDP-43) that forms the cytoplasmic inclusions within frontotemporal dementia (FTD)-amyotrophic lateral sclerosis (ALS) pathologies. We sought to investigate the interaction between the redox forms GRNs-3 and 5 and the toxic C-terminal domain of TDP-43 (TDP-43CTD) and identify the effect of the former two proteins on the stress granule (SG) assembly consisting of TDP-43CTD and RNA.

Methods: These studies were performed in-vitro. Here, the presence of amyloidogenic species of TDP-43CTD as a consequence of its interaction with GRNs 3,5 was confirmed using thioflavin-T (ThT) fluorescence assay. Fluorescence microscopy was used to study the modulation of the liquid-liquid phase separation of TDP-43CTD by GRNs. Matrix assisted laser desorption-ionization (MALDI) time-of-flight (ToF) mass spectrometry was used to characterize the colocalization of proteins under study.

Results: We found that GRN-5, in both redox forms, undergoes LLPS with TDP-43CTD augmenting the aggregation of the latter into ThT positive species, while GRN-3 also promotes the aggregation but without coacervation. GRNs also disrupted the SGs of RNA and TDP-43CTD and initiated the aggregation of the latter.

Conclusion: Overall, we show that both redox forms of GRN-3,5 have pathogenic consequences in their association with TDP-43CTD, albeit via different mechanisms. To the best of our knowledge, this study is one of the first to shed insights into the molecular underpinnings of interaction between GRNs and TDP-43.

Structural Studies of GSS-Associated Y145Stop Prion Protein Amyloids by Solid-State NMR Spectroscopy

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Abstract:

Aims/Purpose: The objective of the study is to investigate the impact of A117V, M129V and A117V/M129V mutations on the human prion fibril formation kinetics and molecular conformation.

Methods: The mutations of interest were incorporated into the huPrP23-144 prion fragment via site-directed mutagenesis and confirmed with Sanger sequencing. The mutated PrP23-144 proteins were expressed in *E. coli* BL21 (DE3) competent cells and purified using FPLC and multiple rounds of dialysis, following previously established protocol. Purified, soluble PrP23-144 proteins were converted into amyloid fibrils within 36 to 48 hours, with 70% or higher conversion rate. The fibrils morphology was studied under atomic force microscopy (AFM), following by structure analysis using solid-state NMR.

Results: Both A117V and M129V mutations have significant impact of the structure of the huPrP23-144 wild-type, producing distinctly different fingerprint 2D NCA NMR spectra. Both mutations effectively shorten the rigid core region of the wild-type fragment down by 10 residues. The double mutant, A117V/M129V fully adopts the structure of the M129V single mutant.

Discussion: Because the double mutant's structure is the same as the M129V single mutant, V129 seems to have a stronger impact on the structure of the amyloid fibril than V117, even though both mutations can alter the structure of the wild-type protein. A117V mutation is reportedly observed along with V129, either as homozygous VV129 or heterozygous MV129, suggesting that the V129 might have some effect on GSS A117V susceptibility. The M129V polymorphism is also observed in other GSS-causing mutation but the role of this polymorphism remains enigmatic.

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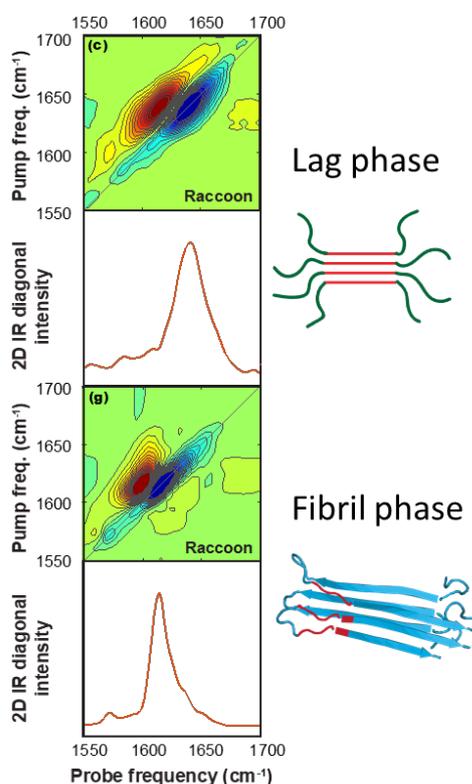
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Animal propensities for diabetes strengthens oligomer hypothesis as shown using 2D IR spectroscopy

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Aims/Purpose: Islet amyloid polypeptide (IAPP) is a 37-residue peptide that misfolds and aggregates into fibrils. These fibrils are deposited as plaques in pancreatic islet tissue in 90-95% of patients with type 2 diabetes. However, studies have shown the fibril itself is non-toxic and an oligomeric transient species is the structure that leads to cell death. Species-dependent sequence differences in residues 20-29 (the “FGAIL region”) appear to dictate the tendency to aggregate in vitro and correlate to the potential to contract type 2 diabetes. Whether each species forms the oligomeric transient species is unknown.

We use two-dimensional infrared (2D IR) spectroscopy and isotope labeling to study the aggregation mechanism of IAPP in real time from seven different animal species. We combine spectroscopy with toxicity assays to determine whether the oligomeric transient species must be present to observe cell death.

Results and Conclusion: For the species susceptible to type 2 diabetes, we observe a transient oligomeric intermediate on route to fibril formation. This oligomeric structure is not observed in IAPP of species that do not

contract amyloid-associated type 2 diabetes. Our toxicity data supports the hypothesis that the oligomeric transient intermediate is the source of cytotoxicity in amylin aggregation.

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Thiol-mediated and catecholamine-enhanced multimerization of a cerebrovascular disease enriched fragment of NOTCH3

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Aims/Purpose. Cerebral small vessel disease (SVD) is a common condition linked to dementia and stroke. As an age-dependent brain pathology, cerebral SVD may share molecular processes with core neurodegenerative diseases such as Alzheimer's and Parkinson's disease. Many neurodegenerative diseases feature abnormal protein accumulation and aberrant protein folding, resulting in multimerization of specific proteins.

Cerebral Autosomal Dominant Arteriopathy with Subcortical Infarcts and Leukoencephalopathy (CADASIL) is the most common genetic cause of SVD, stroke, and vascular dementia¹. CADASIL is characterized by NOTCH3 mutations, NOTCH3 protein accumulation in arteries, and granular osmiophilic material²⁻⁶. The mechanisms underlying CADASIL protein accumulation are currently unknown.

We identified that NOTCH3 undergoes post-translational processing to generate a truncated fragment of NOTCH3 in human CADASIL tissue, which we termed N-terminal fragment (NTF). We test the hypothesis that NTF, which co-registers with pathologically affected cells in the inherited SVD, is capable of multimerization. We also characterized endogenous small molecule vascular enhancers and inhibitors of multimerization.

Methods. We generated rabbit monoclonal antibodies against the N-terminal region of NOTCH3. For protein analysis, NTF was synthesized by ThermoScientific. Lyophilized NTF was reconstituted in dH₂O at 1ug/uL. All protein treatments were performed in PBS with supplemental chemicals from Sigma. Standard Western Blot, Silver Stain (ThermoScientific), and Coomassie Blue (Bio-Rad) staining protocols were used. For mass spectrometry experiments, synthetic NTF was incubated with chemical treatments in dH₂O. Samples were infused into OrbiTrap Fusion Tribid mass spectrometer. The precursor mass (MS1) scans were collected and deconvoluted using the Xtract function of QualBrowser (ThermoScientific, ZSCalibur v3.0.63).

Results. NTF multimerizes spontaneously and also forms conjugates with vascular catecholamines, including dopamine and norepinephrine, which avidly promote multimerization of the protein. Inhibition of catecholamine-dependent multimerization by vitamin C and reversal by reducing agents implicate an essential role of oxidation in NTF multimerization. Antibodies that react with degenerating arteries in CADASIL tissue preferentially bind to multimerized forms of NTF.

Discussion/Conclusion. A large contingent of neurological diseases is thought to result from proteinopathies. Examples include Alzheimer's disease, which features misfolded tau and multimerized amyloid beta protein, Parkinson's disease, which features abnormal conformation, accumulation and multimerization of synuclein, and Creutzfeldt-Jacob Disease, the prototypical propagating proteinopathy^{7,8}. In all these examples, protein abnormalities localize in or around neurons; this proximity to pathology is felt by some to implicate a direct effect of proteinopathy on cell viability.

Several vascular diseases of the brain share similarities to proteinopathies seen in neuronal disorders. Our findings from the sporadic and genetic cerebral SVD, CADASIL (Cerebral Autosomal Dominant Arteriopathy with Subcortical Infarcts and Leukoencephalopathy), demonstrate dramatic NOTCH3 protein accumulation, cleavage, and multimerization of the truncated fragment. Our studies suggest that multimerization of proteins in the aging brain is not restricted to neuronal molecules and may participate in age-dependent vascular pathology.

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Investigating Retinal Blood Flow Characteristics and Amyloid Formation in Patients with Type 2 Diabetes and Mild Cognitive Impairment.

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Purpose: Patients with type 2 diabetes (T2D) have almost a two-fold greater risk of developing Alzheimer's disease. Recent studies suggest that one link between the two diseases may be the processes underlying amyloidosis.¹ Also, our previous studies have demonstrated that cognitive impairment (CI) is not limited to the brain but also affects the retina. In this pilot study, we quantified blood flow within the optic nerve head (ONH) using the XyCAM RI (Vasoptic Medical, Inc.), a non-invasive, laser speckle-based retinal imager, in cognitively healthy subjects and patients with mild cognitive impairment (MCI) and T2D.

Methods: Nine subjects (2 controls, 3 T2D, and 4 MCI) were imaged using the XyCAM RI. Intraocular pressure and blood pressure measurements were obtained before imaging, and heart rate and oxygen saturation were recorded during image acquisition. Four imaging sessions, each of 6 seconds duration, were conducted on both eyes of each subject. Retinal blood flow (RBF) was computed as the mean of all vascular and non-vascular regions in a circular region of interest (ROI) overlying the optic nerve head (ONH) (Figure 1). RBF data corresponding to three complete cardiac cycles without motion artifacts was averaged to obtain trough (diastole), mean (temporal), and peak (systole) RBF estimates. Independent sample t-test and Kruskal-Wallis test were used to determine the significance of differences in RBF estimates between patient type.

Results: Table 1 reports the average RBF over the entire retinal imaging field during systole, diastole and mean; as well as ROI-specific data in the ONH region including the following waveform metrics: S (systole RBF), D1 (first diastole RBF), D2 (second diastole RBF), RM (rising mean RBF), FM (falling mean RBF), TtS (time from first diastole to systole in milliseconds), TtD (time from systole to second diastole in milliseconds), SSVI (systolic stroke volume index), and DSVI (diastolic stroke volume index). The Kruskal-Wallis test was conducted on all metrics to compare group means (Table 1). Patients with MCI and T2D had a higher systolic and diastolic RBF compared to cognitively healthy subjects ($p < 0.01$ and $p = 0.03$ as per the independent sample t-test, respectively). Additionally, as per the independent sample t-test, patients with MCI have a greater ONH RBF in the D2 ($p < 0.01$), RM ($p = 0.01$), FM ($p < 0.01$), SSVI ($p = 0.01$), and DSVI ($p < 0.01$) phases compared to controls. When comparing MCI and T2D groups, MCI subjects had a higher RBF in systole and diastole. At the ONH, MCI subjects had a higher S, D2, RM, FM, TtS, TtD, SSVI, and DSVI compared to T2D subjects (all $p < 0.03$).

Discussion: In conclusion, our pilot study demonstrated that individuals with MCI and T2D have different retinal blood flow characteristics compared to healthy cognitive subjects. Also, it was found that patients with MCI have a higher RBF compared to both T2D and healthy cognitive subjects, and that T2D patients also have a higher RBF compared to healthy cognitive subjects. However, because our study sample was small, the full extent of clinical applicability of our approach is provocative and still to be determined. Non-invasive blood flow imaging of the ONH show promise in potentially distinguishing between patients with T2D and MCI, and should be investigated as a potential biomarker for classifying status and severity of amyloidosis formation. While further studies with an expanded population must be performed to demonstrate further and understand RBF changes in MCI and T2D subjects concerning the processes underlying amyloidosis, this pilot study lays the groundwork for future work in this area.

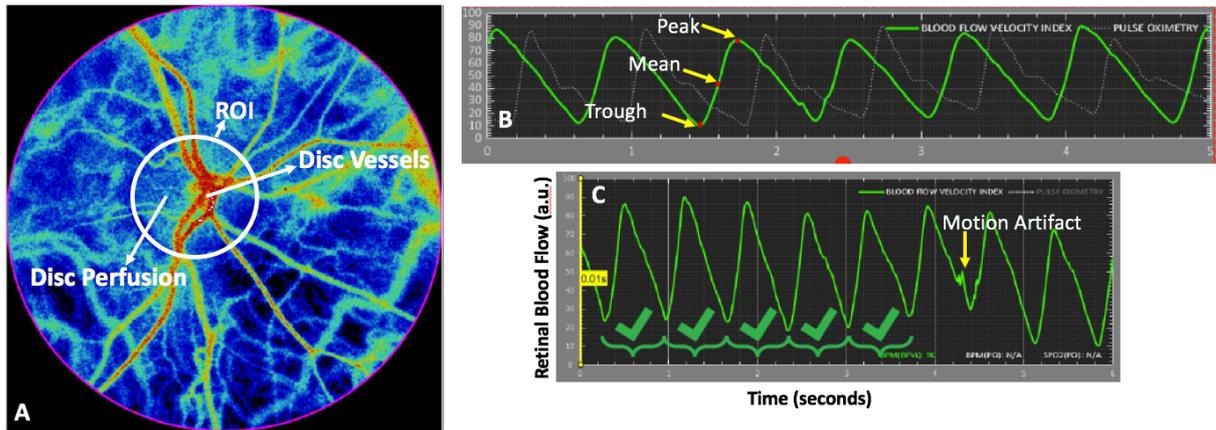


Figure 1. Assessment of retinal blood flow (RBF) over a cardiac cycle in the optic nerve head. (A) An image of the human optic nerve head (ONH) obtained by the XyCAM RI with blood flow in pseudo-color (hotter colors indicate higher flow). Disc perfusion is depicted as the region of the ONH with vessels excluded whereas disc vessels includes all vessels spanning the ONH. (B) Temporal plot of RBF (a.u.) and pulse oximetry waveforms. The RBF waveform illustrates the trough, mean, and peak RBF measurements over a single cardiac cycle. (C) Temporal plot of RBF (a.u.) illustrating examples of acceptable cardiac cycles and motion artifact.

	Overall Field of View			ONH Region								
	Systole	Diastole	Mean	S	D1	D2	RM	FM	TtS	TtD	SSVI	DSVI
MCI	7.8±0.5	6.2±0.8	7.0±0.7	7.7±0.5	6.0±1.6	6.2±0.8	6.8±1.0	7.0±0.6	329.9±104.2	630.7±162.0	2.2±0.5	4.3±0.8
T2D	7.1±0.6	5.5±0.3	6.3±0.4	7.0±0.6	5.4±0.4	5.3±0.4	6.2±0.4	6.1±0.4	259.6±50.7	518.3±127.4	1.6±0.4	3.2±0.8
Controls	6.7±0.3	5.2±0.3	5.9±0.3	6.7±0.3	5.2±0.2	5.2±0.3	5.9±0.3	5.9±0.3	292.6±22.1	552.8±52.2	1.7±0.2	3.3±0.4
Kruskal-Wallis	0.000	0.000	0.000	0.000	0.000	0.001	0.000	0.000	0.023	0.233	0.000	0.000

Table 1. Blood Flow Velocity Index (BFVi in a.u.) results and Kruskal-Wallis test outcomes, reported as p-values. MCI=Mild Cognitive Impairment; T2D= type 2 diabetes; S=Systole; D1=Diastole 1; D2=Diastole 2; RM= Rising Mean; FM= Falling Mean; TtS=Time to Systole; TtD=Time to Diastole; SSVI= systolic stroke volume index; DSVI= diastolic stroke volume index.

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Probing the mechanisms of amyloid-beta fibril formation by NMR

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Aims: The formation of plaques made of long fibers of amyloid-beta has been implicated as one of the possible causes of Alzheimer's Disease. The process by which these fibers grow has been mysterious. In particular, details of how incoming molecules attach to the end of fibers have been inaccessible to experiments. Without atomic-level information, it has been difficult to design inhibitors to block fiber growth. In this study, we demonstrate the feasibility of using NMR experiments to probe the binding process indirectly and show the mechanism is actually a multi-step process in which amyloid-beta initially binds in a conformation distinctly different than the end conformation, giving a potentially new point of attack at blocking fiber growth.

Methods: High-resolution NMR experiments were used to probe the interaction of ¹³C & ¹⁵N-labeled amyloid-beta monomers to preformed structures of amyloid fibrils. The formation of small oligomers and evidence for dock-lock type mechanism of fibril growth are obtained with the use of fibers and protofibrils. Transmission electron microscopy (TEM) images were used to analyze the morphology of fibrillar samples.

Results: There is significant interest in obtaining high-resolution insights into the mechanisms of toxic oligomeric amyloid intermediates formation.[1-3] In this study, we utilize the line-broadening due to exchange between free and fiber-associated monomers of A β ₁₋₄₀ in a sample seeded with amyloid fibers to indirectly observe the docked state. These NMR measurements enabled us to monitor A β aggregation at atomic-resolution without the time constraints, allowing the kinetic processes of fibril assembly to be monitored with residue specificity (Figure 1).[4] While both fibers and protofibrils have loosely bound surface associated states, the binding mode was found to be different.[4, 5] In the protofibril-monomer system, the KLVFFA (residues 16-21) sequence of the A β ₁₋₄₀ monomer has the highest affinity for the protofibril surface. On the other hand, in fully formed fibers, the main recognition sequence of the docked state was found to be centered on the F19-N27 region. This observed shift is significant as the KLVFF sequence has been implicated in A β ₁₋₄₀ self-recognition and modified forms of the peptide sequence have been used for this reason as a motif in the design of peptide inhibitors for arresting fiber formation. Experimental results and a mechanism for oligomer formation and fibril growth will be presented.

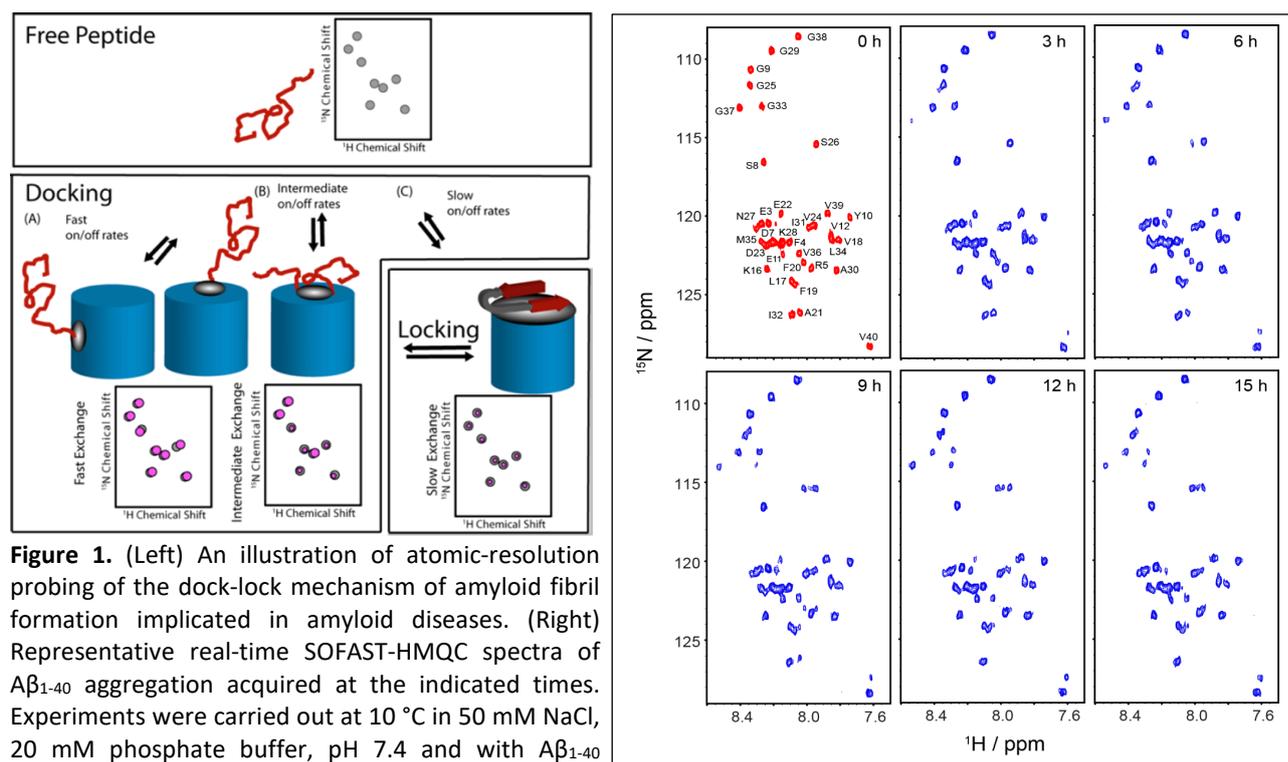


Figure 1. (Left) An illustration of atomic-resolution probing of the dock-lock mechanism of amyloid fibril formation implicated in amyloid diseases. (Right) Representative real-time SOFAST-HMQC spectra of A β ₁₋₄₀ aggregation acquired at the indicated times. Experiments were carried out at 10 °C in 50 mM NaCl, 20 mM phosphate buffer, pH 7.4 and with A β ₁₋₄₀ concentration of 80 μ M.

Conclusion: NMR experiments are used to probe transient binding of amyloid-beta monomers to fibers. Experimental results reveal the formation of partially bound amyloid-beta conformations with the highest degree of interaction near F19-K28 and a lesser degree of interaction near the L34-G37 region. We believe that the NMR approach and the findings will be useful to monitor the formation of intermediate amyloid species which can provide mechanistic insights to explain the fibril polymorphism and could aid in the development of efficient inhibitors.

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Amyloid- β 40 Nucleation – In Two Directions

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Aims/Purpose: Our aim in these studies is to understand the early events in protein aggregation leading to amyloid fibril formation. Polymorphism of protein aggregates has been well established for amyloid fibrils, but probably arises in earlier stages such as nucleation. This conclusion is supported by the fact that for amyloid- β 40 (A β 40), seeding disaggregated solutions of A β 40 with fibril seeds leads to the formation of *replicate fibrils*, i.e., by adding seeds, one bypasses spontaneous nucleation steps. Our goal in the present studies is to use solution and solid-state NMR, atomic force microscopy, fibril x-ray diffraction, and other biophysical methods to understand these nucleation and seeding events.

Methods: 1) To study the early events in nucleation, we are using solution and solid-state NMR, especially paramagnetic relaxation enhancement (PRE) methods, in combination with atomic force microscopy (AFM) and other biophysical techniques. 2) In another set of studies, we examined the effects of added seeds. Using previously described methods (1-3), we generated replicate fibrils made of ¹⁵N and ¹³C-labeled, synthetic A β 40, by seeding with amyloid material isolated from the brains of patients with Alzheimer's Disease (AD) and/or Cerebrovascular Amyloid Angiopathy (CAA). We then compared solid-state NMR spectra and fibril x-ray diffraction patterns of brain parenchyma-seeded replicate fibrils vs. replicate fibrils seeded from cerebrovascular amyloid obtained from meninges.

Results and Conclusions: 1) Using NMR, AFM, and other biophysical methods, we have shown that A β 40 forms flickering aggregates that rapidly form and dissociate, and which we call NanoDroplet Oligomers (NanDOs). NanDOs were first observed using video-scanning rate atomic force microscopy (VRS-AFM), and were subsequently studied by PRE. PRE suggests that NanDOs form mainly due to the hydrophobic effect, i.e., transient interactions mainly among hydrophobic amino acid side chains. Additional studies indicate that addition of divalent metal ions (Zn²⁺) leads to further aggregation of NanDOs through flocculation. 2) A comparison of brain parenchyma- and cerebrovascular-seeded replicate A β 40 fibrils by solid-state NMR, fibril x-ray diffraction, and other biophysical techniques indicates significant differences between the two types of fibrils. In particular, two-dimensional ¹³C-¹³C correlation and other solid-state NMR spectra indicated differences, especially within the N-terminal domain, between side chain chemical shifts of parenchyma- vs. vascular-seeded fibrils. Fibril x-ray diffraction studies showed typical reflections at ~5 and ~10-11 Å, but also indicated that vascular-seeded fibrils had a greater level of order in the ~10-11Å reflection. We conclude that there are structural differences in the A β fibrils obtained for seeding from parenchyma plaques vs. cerebral blood vessel sources.

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Toxicity and Characteristics of Monomer vs. Fibril-derived Oligomers

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Abstract: There is compelling evidence that small oligomeric aggregates, emerging during the assembly of amyloid fibrils and plaques, are the dominant pathogenic species of amyloid diseases.[1-4] While significant progress has been made in revealing the mechanisms underlying fibril growth,[5] the question of how amyloid oligomers fit into the fibril assembly process has remained controversial. Commonly, amyloid oligomers are considered metastable, early-stage precursors that are either on- or off-pathway from fibril growth. [5-8] At the same time, late-stage fibrils and plaques are reported to be reservoirs of amyloid oligomers. These two viewpoints, however, are at odds with each other: metastable oligomers should only form transiently during assembly instead of re-emerging as products of fibril dissociation reactions. Elucidating the relation of oligomer to fibril formation has important implications for our understanding of the role of oligomers in the pathogenesis of amyloid diseases.

Investigating the toxicity of early-stage oligomers vs. late-stage fibrils, formed *in vitro* from hen egg-white (hewL) lysozyme, we were confounded by the observation that fibrils were significantly more toxic than early stage oligomers. Control experiments revealed, though, that fibril samples contained highly toxic admixtures of oligomeric species. These fibril-associated oligomers had morphologies and tinctorial features comparable to monomer-derived oligomers. However, they were distinct in their β -sheet content, their stability and biological activity. Our observations suggest that different categories of amyloid oligomers emerge via assembly from either monomers or from dissociation from fibrils.

Results: We have previously reported that amyloid assembly of lysozyme can switch between two distinct assembly pathways. A “pure rigid fibril (RF)” pathway is characterized by sigmoidal ThT kinetics with no detectable populations of metastable oligomeric precursors formed during the lag phase. Upon crossing a threshold in protein (or salt) concentration, ThT kinetics turn progressively more biphasic with prominent populations of metastable globular oligomers (gOs) and curvilinear fibrils (CFs) emerging without any delay.[9]

Here we investigated the cell toxicity of the fibril and oligomer species emerging along these two pathways. Either pure fibril or early-phase oligomer samples were grown under nearly identical solution conditions (20 mg/ml hewL, pH 2, T = 52 C) but in the presence of either 50 mM or 250 mM NaCl (i.e. fibril vs. oligomeric pathway). Aggregation was monitored using dynamic light scattering and the reaction stopped at appropriate time points to obtain suspensions of RFs or gO/CFs.

Cell viability measurements of adeno-carcinomic human alveolar basal epithelial (A549) cells were performed using an imaging live-dead fluorescence assay. Upon growing cells to confluency, the cells were exposed to either RF or gO/CF preparations. Dilutions of aggregate samples were chosen so that the aggregate growth media (50 or 250 mM NaCl) at equivalent concentrations of added hewL monomers did not affect cell viability. Cells were exposed for 48 or 72 hours to RF and gO/CF samples at concentrations ranging from 1 up to 100 μ M.

Using microscope imaging to evaluate cell toxicity revealed that the transfer of the *in vitro* aggregates from pH 2 into the pH 7.4 cell medium induced the formation of large macroscopic assemblies. RFs assembled into large sheets of bundled fibrils which displayed the prominent birefringence characteristics of amyloid plaques. GO/CFs, in contrast, precipitated into gel-like, diffuse clusters. Counter to our expectations, RFs even at 1 μ M concentration caused a significant 20% drop in cell viability while gO/CFs even at the highest concentration of 100 μ M reduced cell viability by less than 10%.

To exclude that RFs solutions contained small aggregates responsible for the observed fibril toxicity, we centrifuged RF samples overnight at 15,000 g and 10 °C. The resulting supernatant was decanted and preserved, and the fibril pellet resuspended in growth solution. To our surprise, it was the RF supernatant instead of the RF pellet that caused the most severe toxicity. In addition, only the RF

supernatant replicated the prominent changes in cell morphology originally observed in the unseparated RF experiments

Subsequent experiments indicated that fibril-associated small aggregates shared many characteristics of monomer-derived oligomers, albeit with some important differences. Solution transfer of the RF supernatant to cell growth medium, for one, induce a similar slow assembly into macroscopic structure with the morphologies identical to those formed by early-stage oligomers. The fluorescence of ThT and the recently identified oligomer-selective dye crystal violet [10] were noticeably enhanced by these fibril-associated oligomers. In addition, both monomer-derived and fibril-associated oligomers displayed the characteristic amyloid peak and an antiparallel β -sheet peak in the amide-I bands of their FTIR spectra. However, the antiparallel β -sheet peak was much more prominent in the fibril-associated oligomers. Equally important, fibril-associated oligomers were in stable equilibrium with RFs, while RFs readily dissociate monomer-derived oligomers. Our preliminary findings indicate that these oligomers emerge as dissociation product from mature RFs and display characteristics similar to, yet distinct in important aspects, from the monomer-derived oligomers formed prior to RF nucleation.

Discussion: Our observations suggest that amyloid formation can result in at least two distinct classes of oligomers emerging either *via* direct assembly from monomers or from dissociation of late-stage fibrils. This could resolve some of the apparent contradictions of oligomers categorization as either early-stage metastable precursors or late-stage dissociation products of fibrils. Equally important, while both classes of oligomers are weakly ThT positive and display comparable morphologies, they differ in their structural details, stabilities and biological activity. The formation of distinct oligomer species from fibrils might also relate to the observation of fibril polymorphs. Different fibril polymorphs might differ either in the rate of oligomer formation or generate oligomers with different structures. In either case, our observations suggest that well-controlled *in vitro* studies of amyloid formation are critically important for unraveling the complexities of amyloid assembly and its relation to the clinical symptoms of amyloidoses.

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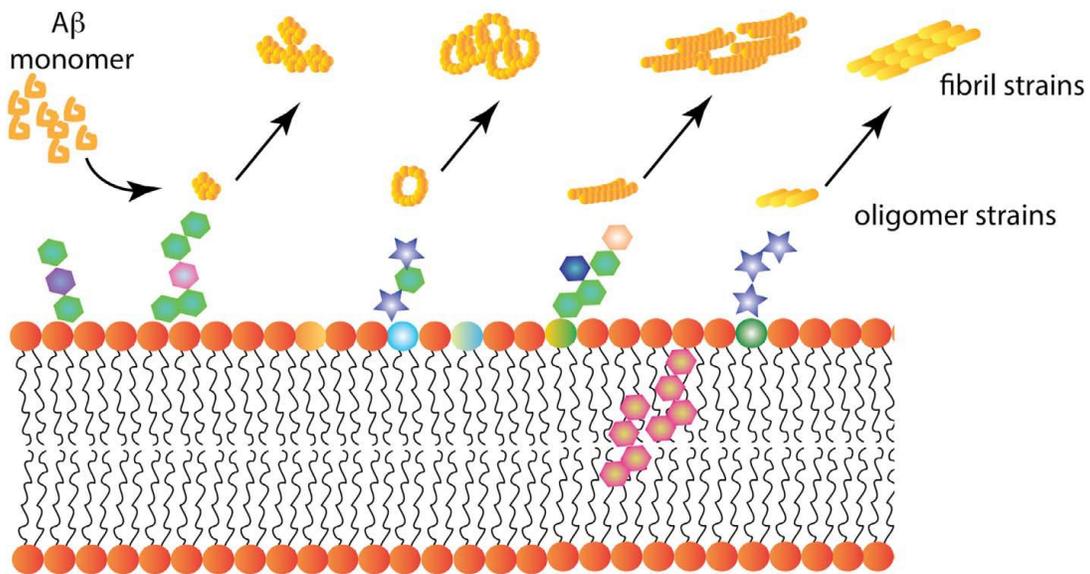
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How do lipid interfaces affect A β oligomer strain generation?

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The emergence of many clinical sub-types has become a distinguishing feature among many Alzheimer disease (AD) patients. Deciphering the molecular underpinnings that govern the phenotype diversity for an idiopathic disease such as AD, is rather challenging. However, correlation between the polymorphism among amyloid- β (A β) aggregates and clinically observed pathologic variations, has in part, corroborated the emerging hypothesis that conformational differences among A β aggregates could dictate phenotypic outcomes. In our lab, we are interested in understanding how different strains of A β are generated, and how such strains end up with conspicuous pathological features. We hypothesize that among various interacting partners, lipid surfaces and interfaces contribute to the diversity in A β conformers at early stages of aggregation, which in turn modulates the outcome of the pathways and concomitant structure and morphology. Using a combination of biophysical, biochemical and computational investigations, we demonstrate that oligomeric strains of A β are generated by varying lipid compositions, and such neurotoxic oligomers propagate their mesoscopic structure towards morphologically unique fibrils. Furthermore, lipid-induced oligomers induce selective phenotypes transgenic mice brains that are different from those of unseeded or fibril-seeded aggregates. These results bring forth important mechanistic insights into the potential role of lipids in catalyzing oligomer strains of A β , and their induction of specific phenotypes.



Why are the populations of amyloid oligomers so high? And how we might use that information to design a mouse model?

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Many amyloid proteins form toxic oligomers during the lag phase of aggregation, prior to fibril formation. This talk will present evidence for an intermediate in the aggregation pathway of amylin from type 2 diabetes. We show that this intermediate is created by a free energy barrier set by a conformational transition between a folded and unfolded protein structure. That transition kinetically traps the oligomer, prolonging the lifetime and creating a stable population. We propose a strategy for trapping this oligomer by destabilizing the fibril structure, thereby indefinitely prolonging the conversion of oligomers to fibrils. Moreover, that information might be used to create a new transgenic mouse model specific for toxic oligomers. While our data and experiments are specific to amylin, the idea might be applicable to other amyloid diseases as well.

Can we target A β oligomers for early diagnosis of Alzheimer's disease? Shai Rahimipour

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Abstract:

Alzheimer's disease (AD) is an incurable neurodegenerative disease, leading to cognitive impairment and progressive synaptic damage accompanied by neuronal loss. Misfolding of A β and tau proteins and their aggregation and accumulation are the main hallmark of AD. Recent clinical studies collectively suggest the necessity of diagnosing AD at an early stage for effective therapy. However, in spite of significant progress in imaging technologies, early diagnosis of AD remains a major challenge, which impedes successful treatment. We have previously reported on self-assembled cyclic D,L- α -peptides that can cross-interact with different amyloidogenic proteins and modulate their aggregation and toxicity by interacting with their early oligomers, most probably due to their structural and functional similarities to those of amyloidogenic proteins. For example, a cyclic D,L- α -peptide recognized by the conformational antibody A11, CP-2, cross-reacts with A β , α -synuclein and tau-derived peptide and modulates their aggregation and toxicity through an "off-pathway" mechanism.

Aims:

In this study, we present a novel diagnostic strategy for molecular computed tomography (CT) imaging of A β aggregates at an early stage of AD. This strategy is based on the development of blood brain barrier (BBB)-permeable gold nanoparticles (GNPs) and a novel self-assembled cyclic D,L- α -peptide that selectively targets early A β oligomers, and modulates their aggregation and toxicity.

Methods:

We synthesized 20 nm GNPs whose surface was modified with a vector to increase BBB permeability and a synthetic cyclic D,L- α -peptide to target toxic A β oligomers and plaques. The particles were characterized by their zeta potential and size, TEM and UV/Vis spectroscopy, while their anti-amyloidogenic activity was evaluated using thioflavin T, electron microscopy, immunohistochemistry and cell-based assays. The capability of the particles to accumulate selectively in 5xFAD transgenic mice brains was determined by CT and fluorescent imaging, and further confirmed by inductively coupled plasma (ICP) spectroscopy.

Results:

The ThT, TEM and cell toxicity experiments demonstrated that the conjugated GNPs preserved the original properties of the anti-amyloidogenic cyclic D,L- α -peptide to bind and inhibit A β aggregation and to decrease its toxicity to neuron-like cells. In unfixed hippocampal coronal slices derived from 6-months old transgenic 5xFAD, the fluorescently labeled conjugated GNPs stained A β species very similar to 6E10 anti-A β antibody, and significantly reduced the amount of plaques following their incubation with the brain slices.

In our *in vivo* CT-based studies, the particles crossed the BBB of both WT and 5xFAD mice and accumulated in their brains 2 hours post intravenous (i.v.) injection. However, 6 hours post injection, the CT signals obtained from 4-months AD animals were significantly more intense than those obtained from WT animals, suggesting that the particles bind A β species and accumulate in AD brains, while are cleared from those of normal brains. These results were also confirmed postmortem by analyzing the gold content in treated brains, using ICP-MS. Most importantly, CT, fluorescent imaging and ICP studies performed following i.v. injection of fluorescently labeled particles,

collectively suggested that AD pathology could be diagnosed even in 2-months old AD animals with no or very low accumulation of A β fibrils and plaques, suggesting that cyclic-D,L- α -peptide-conjugated GNPs indeed bind early A β oligomers.

Conclusion:

Our studies suggest that targeting the common structural conformation of amyloids may be a promising approach for developing new theranostics for amyloidogenic diseases.

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Inhibition of amyloid self-assembly by liquid-liquid phase separation

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Abstract

Designed peptides derived from the cross-amyloid interaction surface of IAPP with A β (interaction surface mimics or ISMs) have been found to be highly potent inhibitors of A β amyloidogenesis and cytotoxicity. Using solution-state and solid-state NMR in combination with ensemble averaged dynamics simulations and other biophysical methods including TEM, fluorescence spectroscopy and microscopy, and DLS, we characterize the structural preferences of the ISM R3-GI and interactions of A β 40 with ISM K3-L3-K3-GI. We find that R3-GI is highly dynamic, oligomerizes into macroscopic structures and undergoes liquid-liquid phase separation. The particle structure is reminiscent of stress granules that are formed by low complexity containing protein domains in vivo. Our findings suggest that ISMs deploy their amyloid inhibitory function via formation of colloidal-like structures which recruit misfolded A β 40 thus preventing its further aggregation and related neurotoxicity.

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Molecular analysis of the transformation from innocuous monomeric to disease-associated fibrillar α -synuclein

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Abstract: Accumulation of α -synuclein aggregates constitutes the hallmark of synucleinopathies including Parkinson's disease. However, many steps from the innocuous, monomeric α -synuclein toward misfolded oligomers and fibrillar species remain unclear. In addition, the interaction mechanism of small molecules, which modulate the aggregation of α -synuclein into amyloid fibrils, is often enigmatic. The combination of NMR spectroscopy with other biophysical experiments and molecular dynamics simulations of α -synuclein provide a comprehensive picture of its conformational dynamics in the monomeric state. The small molecule phthalocyanine tetrasulfonate binds to the dynamic ensemble of monomeric α -synuclein and remodels the conformational landscape resulting in the formation of α -helical oligomers, which are off-pathway to fibrillization. In contrast to several other small molecules, phthalocyanine tetrasulfonate also inhibits the aggregation of α -synuclein in the presence of a membrane environment. Phthalocyanine tetrasulfonate directly binds to vesicle-bound α -synuclein, stabilizes its helical conformation and thereby delays pathogenic misfolding and aggregation. The combined data suggest that compound-related stabilization of helical vesicle-bound α -synuclein opens new possibilities to target Parkinson's disease and related synucleinopathies.

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In cell structural biology of metastable proteins using sensitivity-enhanced NMR
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Abstract:

Aims & Purpose: For a cell to survive, its proteins must adopt their functional conformations. For many proteins, this conformation is highly energetically favored. However, some proteins adopt more than one conformation. In such cases, their environment will influence which conformations such proteins adopt. Thus, we seek to determine protein structures of conformationally plastic proteins in their native environments: inside cells. To do so, we use DNP-enhanced magic angle spinning (MAS) NMR spectroscopy. MAS NMR is not size-limited and can be used to study membrane bound proteins, protein oligomers and even megadalton-size molecular complexes like amyloids. The main limitation of using MAS NMR spectroscopy for in-cell studies is sensitivity. We find that DNP increases the sensitivity of MAS NMR experiments enough to permit detection of proteins at their endogenous (micromolar) concentrations inside intact frozen cells.

Methods: Sensitivity and specificity are crucial if we want to study proteins at physiological concentrations inside intact cells. Dynamic nuclear polarization (DNP)-enhanced MAS NMR spectroscopy provides sufficient sensitivity to allow in-cell measurements at physiological concentrations (1,2). Specificity can be achieved by introducing purified, isotopically labeled protein into cells using well-described electroporation approaches (3). DNP MAS NMR experiments are performed at cryogenic temperatures. Low temperatures enable long measurements on intact cells without any impact on cellular viability or integrity provided no damage occurs during freezing. We developed methods that support DNP enhanced MAS NMR data collection on intact cells that maintain viability throughout the experimental time course.

Discussion: We applied our methodology to determine the conformations adopted by the intrinsically disordered protein, α -synuclein inside viable mammalian cells. This protein can adopt α -helical, random coil and multiple beta-sheet-rich conformations. We apply this approach to mammalian cells into which isotopically enriched α -synuclein was introduced and determine conformational ensembles of this intrinsically disordered protein inside of viable cells. Measurements at cryogenic temperatures that are non-destructive in nature open doors to new applications such as examination of cell phenotypes before and after data collection with the goal of correlating biological phenotypes directly to NMR-based experimentally determined structural ensembles.

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Amyloid fibrils from the N-terminal prion protein fragment as a model for studying the mechanism of prion propagation and transmissibility barriers

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Background: Prions are infectious amyloids that cause a group of transmissible neurodegenerative diseases (known as transmissible spongiform encephalopathies) that include Creutzfeldt-Jakob disease in humans, scrapie in sheep, bovine spongiform encephalopathy in cattle, and chronic wasting disease in deer and elk. These unusual pathogens are believed to propagate by the templated (seeded) conversion of the normal prion protein (PrP) to the amyloid form. The mechanistic and structural aspects of prion protein conversion to the infectious form are, however, poorly understood. Little is also known about the structural basis of prion strains and transmissibility barriers.

Results and Discussion: Recently we have shown that amyloid fibrils from the N-terminal part of the recombinant mouse prion protein (PrP23-144) are infectious, causing transmissible prion disease in mice. Thus, these fibrils which, unlike other mammalian prions, are amenable to detailed biophysical and structural analysis, are of unique value as a model for exploring the mechanism of prion protein conformational conversion as well as the molecular and structural basis of prion strains and transmissibility barriers. Here we will describe a series of low- and high-resolution biophysical experiments, including solid-state NMR spectroscopy, with PrP23-144 amyloids from three species: human, mouse and Syrian hamster. Our data show that these fibrils adopt different structures, and these species-dependent structural differences are controlled by only two residues within the amyloid core region. Importantly, species-specific structural differences correlate with seeding specificities of PrP23-144 amyloid fibrils. The role of these critical amino acid residues as conformational switches can be rationalized based on the structural model for human PrP23-144 amyloid fibrils, providing a foundation for understanding cross-seeding specificity.

Superfamily of β -solenoid amyloids reveals determinants of prion seeding specificity

Antoine Loquet

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Abstract: The amyloid fold is a generic β -sheet-rich protein fold involved in neurodegenerative diseases but also displaying various cell functions in animals, fungi and bacteria. Herein, we explore the sequence-to-fold relation in amyloids. We compare a novel prion termed HELLF to the HET-s prion model using solid-state NMR. We find that these two amyloids sharing only 17% sequence identity have nearly identical β -solenoid folds but lack cross-seeding ability, indicating that prion specificity can differ in extremely similar amyloid folds. We then use protein design to explore the extreme limit of the sequence-to-fold conversation and to pinpoint determinants of prion seeding specificity. We find that β -solenoid fold conservation occurs even at an exceedingly low level of identity to HET-s (5%) and design a structurally promiscuous prion overcoming the seeding barrier to HET-s. Our results reveal the extensive evolutionary diversification of the β -solenoid fold into structurally similar but functionally distinct prion amyloids.

Role of Water in Protein Aggregation and Amyloid Polymorphism

Dave Thirumalai

The University of Texas at Austin

Abstract:

I will discuss the role water plays in the mechanism of protofilament formation from peptides yeast prion peptide and A β peptides as well how water molecules play an important role in amyloid polymorphism. I will show that spontaneous formation of two-ordered one-dimensional water wires in the pore between the two sheets of the Sup35 protofilaments results in long-lived structures, which are stabilized by a network of hydrogen bonds between the water molecules in the wires and the polar side chains. This behavior will be contrasted with A β peptides in which expulsion of water between two sheets is rapid.

Amyloid aggregation simulations: what can we learn from them?

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Aims: The ultimate goal of the studies performed by my research group is to understand why the Alzheimer's peptides A β (1-40) and A β (1-42) start aggregating and how the resulting oligomers look like. We further wish to understand how this aggregation process is altered by the various conditions and components that are found *in vivo*, which is the brain extracellular matrix given the fact that extracellular A β aggregates are a hallmark of Alzheimer's disease.

Methods: We use all-atom molecular dynamics (MD) simulations in their full glory – from ordinary to tricky enhanced simulations – and develop cutting-edge analysis methods to derive as much information as possible from our MD simulations of A β . An important aspect of our work is to compare our simulation results to experimental findings, which enables the interpretation of experimental observations based on A β conformations determined *in silico*.

Results: I will present various results that we obtained from our simulations in the past 3 years. First, I will demonstrate for A β (1-40) and A β (1-42) how transition networks for aggregation that were developed by my lab are able to unequivocally elucidate amyloid aggregation pathways.¹ These networks revealed the importance of elongated oligomers with many hydrophobic residues on their surface in driving the aggregation process. As our long-term goal is to elucidate how the aggregation of A β takes place under *in vivo* conditions,² I will also show how the presence of transition metal ions, oxidative stress and the neuronal cell membrane lead to changes in the structural dynamics and oligomerization of A β .³⁻⁵

Conclusion: The quality and possible length scale of nowadays all-atom MD simulations allow to connect simulation and experiments of A β aggregation. However, from more than a decade of experience in modeling A β I also identified various challenges for these simulations, which I call the force field challenge, the concentration challenge, and the length- and time-scale challenge.⁶ As I want to raise the awareness in the field for these challenges, which limit the conclusions that can be drawn from the amyloid aggregation simulations, I will conclude my talk discussing these challenges.

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The interplay between metals, peptides and amyloids: mechanisms and therapeutic strategies

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Abstract:

Aims: Elucidate the effect of metals in amyloid aggregation and the functional activity of peptides in amyloid aggregation in presence of metals

Methods: Molecular modeling tools and all atom explicit molecular dynamics simulations had been implemented to investigate metal-amyloid aggregates and to study peptides as metal chelators in amyloid aggregates.

Results: The result that will be presented will focus on Insulin. Our work presents for the first time the three-dimensional structures of insulin aggregates and metal-Insulin aggregates. Several Zn^{2+} -binding sites were found in Insulin aggregates. Finally, a few short peptides demonstrated high Zn^{2+} -binding affinity, therefore these peptides may be good metal chelators.

Conclusions: The process in which metals bind to amyloids yielding to a range of molecular mechanism pathways to produce polymorphic metal-amyloid aggregates. Therefore, it is necessary to develop a peptide to inhibit the formation of all polymorphic metal-amyloid aggregates.

Theoretical and computational analysis of the amyloid aggregation kinetics

Prem Chapagain

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Abstract

Amyloid fibrils are a common component in many debilitating human neurological diseases such as Alzheimer's, Parkinson's, and Creutzfeldt-Jakob disease. Over the last two decades, significant progress has been made in elucidating the molecular and cellular processes leading to neurodegeneration and neuronal cell death in these disorders. However, the nature of neurodegeneration is notoriously complex, with heterogeneity in molecular events and mechanisms. Despite the availability of extensive data and both in vitro and animal models, the mechanisms of aggregate initiation and propagation are still being explored. In this respect, theoretical models and computational simulations can complement experiments and provide a detailed molecular-level understanding of the fibrillogenesis process and its role in neurodegeneration, allowing crucial insights needed for developing methods to slow down or prevent these horrific diseases. Based upon the experimental data, we developed mathematical models to determine viable reaction pathways and rate parameters for peptide secondary structural conversion and aggregation during the entire fibrillogenesis process from random coil to mature fibrils, including the species that accelerate the conversions. Separately, we have performed molecular simulations to investigate the nucleation and propagation of fibrils in amyloidogenic peptides. We have found that the structural transitions involve breaking of intrachain hydrogen bonds, and the subsequent cooperative formation of interchain hydrogen-bonds during aggregation into amyloid fibrils. The use and prospects of both the mathematical modeling approach as well as molecular simulations to understand the mechanisms underlying the amyloid fibril initiation, propagation or intervention will be discussed.

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Extra-islet amyloid plaques found in pancreas of transgenic mice and humans using 2DIR microscopy.

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Abstract:

I will present recent work applying 2DIR spectroscopy to generate images of pancreatic mouse tissue. Overexpression of human islet amyloid polypeptide (hIAPP, or amylin) in mice results in hyperglycemia after 6-8 weeks, which is the signature for the onset of type 2 diabetes. It has been previously shown with stains and TEM that hIAPP aggregates into fibrillous amyloid plaques in the islets within the pancreas. Using 2DIR imaging, we report that amyloid deposition occurs throughout the pancreas, not only in the islets. This determination was made via the characteristic infrared absorption of amyloid at 1620 cm⁻¹, small anharmonicities, and cross peaks. Images will be shown for wild-type mice, transgenic diabetic mice, and human tissue. Our work has important implications for the disease, calls into question the interpretation of fluorescence measurements, and establishes 2DIR microscopy as an insightful tool for tissue imaging.

Aims:

The aims of the project are to

- 1.) Develop and apply 2DIR microscopy to mouse tissues to provide a molecular-level understanding of type 2 diabetes, an amyloid disease.
- 2.) Use the findings from **Aim #1** to provide mechanistic insights into diabetes progression in humans. Apply 2DIR microscopy to human tissues and isolated islets.

Methods:

Tissues are extracted from healthy and diabetic mice; human tissues are ordered from organ repositories. We generate hyperspectral images by rapidly scanning 2DIR spectra across each tissue slice. 2DIR parameters such as diagonal peak location, cross peak location, and anharmonic shift are used to clearly demonstrate the presence or absence of amyloid at each location in a tissue.

Results:

Figure 1 displays diagonal slice information from a pancreas removed from a healthy mouse and a pancreas removed from a diabetic mouse. The diabetic pancreas shows clear differences in protein secondary structure compared to the healthy pancreas, which we contribute to amyloid structures in the tissue.

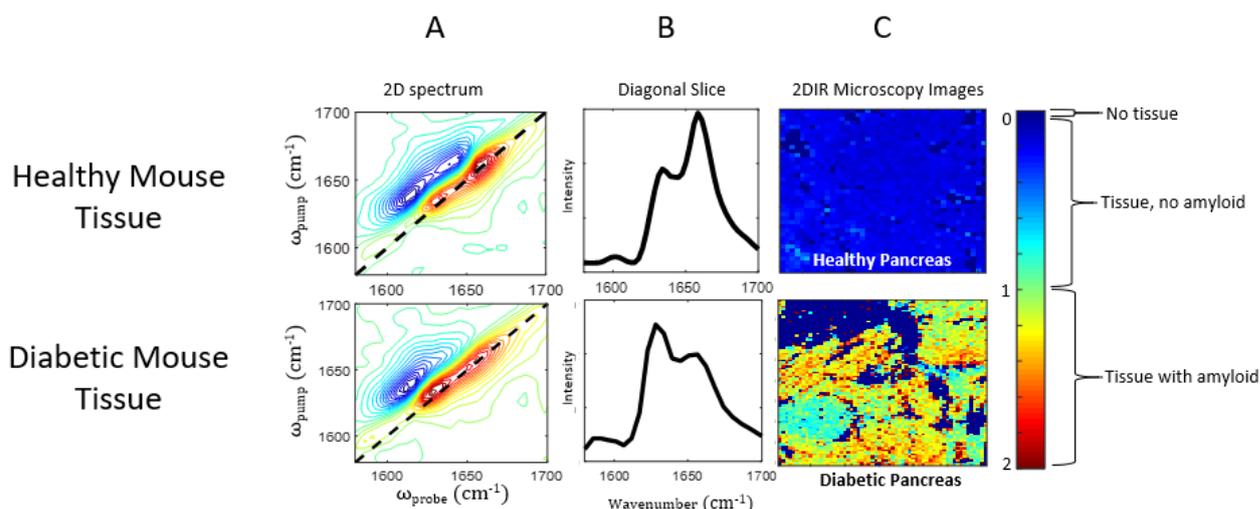


Figure 1: 2DIR microscopy reveals novel insights in protein structure within pancreas tissue. Panel A: Example 2D spectra with diagonal slices through the ground state excitation/bleach (red circles)

in 2D spectrum). Panel B: Diagonal slices from the spectra in Panel A. Panel C: Thousands of spectra are reconstructed into an image of the original tissue and examined for the presence of amyloid.

Discussion:

Pancreatic islets play an important role in the maintenance of healthy blood glucose levels. β -cells within the islets secrete insulin, which stimulates the uptake of glucose from the bloodstream, and amylin, which acts as an appetite suppressant. During type 2 diabetes, amylin misfolds into toxic oligomers; these oligomers harm the β -cell membrane and have well characterized toxic effects. Eventually, these oligomers aggregate into insoluble amyloid plaques, observed by fluorescence microscopy and TEM to occur within pancreatic islets. However, islets constitute only ~2% of the mature pancreas. Outside of the islets, acinar cells secrete enzymes to aid in the digestion of food. Toxicity mechanisms that impact acinar cells have not been well characterized because plaque burden has not previously been associated with these cells and is invisible to other microscopies. In this study, we use 2DIR microscopy to demonstrate that amylin plaques are present throughout acinar tissues; this finding holds important implications for type 2 diabetes and changes our understanding of how amylin impacts pancreatic function.

Two-dimensional infrared (2DIR) spectroscopy is an excellent tool for protein secondary structure determination.^{1,2,3} Novel applications of 2DIR spectroscopy performed in our lab have provided residue-specific structural information on kinetically evolving species¹ and have revealed structural changes in eye lens tissue invisible to standard microscopic techniques.² Here, we have developed and extended 2DIR spectroscopy to microscopy for protein structural imaging. This exciting new method has huge implications for the study of amyloid diseases because amyloid structures are readily identified by our technique. Information revealed by 2DIR microscopy on mouse and human tissues has exciting applications for animal models and therapeutics.

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Tau Amyloid Fibrils Structure and Dynamics from Solid-State NMR

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Abstract:

Tau proteins are intrinsically disordered proteins that stabilize microtubules and are most abundant in neurons of the central nervous system. A group of neurodegenerative diseases, including Alzheimer's disease (AD), are associated with misfolding of tau into fibrillar aggregates. Tau fibrils extracted from postmortem brains of patients with AD and Pick's disease show disease-specific fibril morphologies. In comparison, a recent study of recombinant 2N4R tau fibrillized *in vitro* using heparin found polymorphic morphologies. Elucidating the structure of the tau amyloid fibrils and the misfolding mechanism of otherwise highly soluble tau proteins is crucial to understand the molecular driving force in these diseases and to develop pharmaceutical interventions.

We have investigated the three-dimensional fold and dynamics of heparin-fibrillized full-length 0N4R tau using solid-state NMR spectroscopy. Multidimensional ¹⁵N-¹³C and ¹³C-¹³C correlation NMR spectra revealed the 0N4R tau fibrils to be highly homogeneous and adopt a unique conformation with a hairpin-shaped β -sheet core, which differs from previously reported structures. The tau fibrils exhibit heterogeneous dynamics: in addition to the rigid β -sheet core, semirigid, partially mobile and nearly isotropically mobile domains are observed and distinguished using various solid-state NMR experiments. Water-edited spectra revealed the hydrated residues, shedding light on the protein interaction with water. These results present a comprehensive view of the 0N4R tau fibrils structure and dynamics, and should facilitate future studies of the molecular mechanism of tau misfolding.

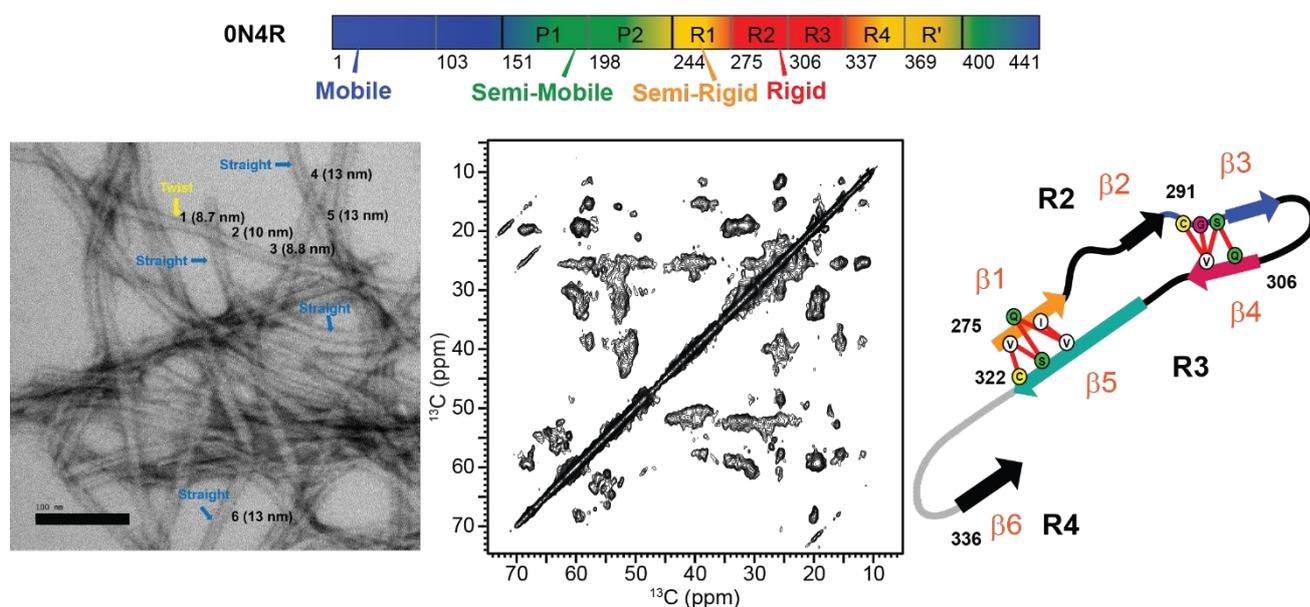


Figure: Left: representative negative-stain TEM image of 0N4R tau fibrils showing predominantly straight filaments; Middle: 2D ¹³C-¹³C correlation spectrum of the fibril; Right: schematic of the β -strand segments and the long-range correlations measured in the SSNMR spectra, defining a hairpin fold for the rigid R2-R3 core.

Tau drives translational selectivity by interacting with ribosomal proteins

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Purpose: There is a fundamental gap in understanding the consequences of tau-ribosome interactions. Tau oligomers and filaments hinder protein synthesis in vitro, and they associate strongly with ribosomes in vivo. Here, we investigated the consequences of tau interactions with ribosomes to assess the role of tau as a direct modulator of ribosomal selectivity.

Methods: We performed microarrays and a novel nascent proteomics method to measure changes in protein synthesis in a progressive mouse model of tauopathy (FTD). We validated these effects in cell culture models and provide the first mechanistic evidence behind tau-mediated ribosomal dysfunction using human post-mortem AD and non-demented control brain tissue.

Results: Using doxycycline-regulatable rTg4510 tau transgenic mice, we determined that tau expression differentially shifts both the transcriptome and the nascent proteome, and that the synthesis of ribosomal proteins is reversibly dependent on tau levels. We further extended these results to human brains and found that tau pathologically interacts with ribosomal protein S6 (rpS6 or S6), a crucial regulator of translation. Consequently, protein synthesis under translational control of rpS6 was reduced under tauopathic conditions in Alzheimer's disease brains.

Conclusions: Our data establish tau as a driver of RNA translation selectivity. Moreover, since regulation of protein synthesis is critical for learning and memory, aberrant tau-ribosome interactions in disease could at least partly explain the linkage between tauopathies and cognitive impairment.

Investigating distinct primary nucleation of A β ₁₋₄₂ polymorphic dimers Olga Press-Sandler^{1,2} and Yifat Miller^{1,2}

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Abstract:

Background: Polymorphic A β dimers are the smallest toxic species that play role in the pathology of Alzheimer's disease. There is a great interest in understanding the malfunctions that yield to these toxic species and to provide insights into the molecular mechanisms of the primary nucleation that can assist in developing effective inhibitors for the formation of these oligomers.

Aim: To investigate the molecular mechanisms of the initial seeding in polymorphic A β ₁₋₄₂ dimers.

Method: Herein, we present a first work that demonstrate two distant edges states of A β dimers. The first is the so called "random coil" state dimer that mimics the primary seeding/nucleation that is far from a fibrillation state. The second is the "fibril-like" state dimer that is structurally in close proximity to fibril, well-organized state into a fibril-like structure. Four polymorphic A β ₁₋₄₂ dimer structures were investigated applying a total of 800ns of all-atom explicit molecular dynamics (MD) simulations: two models of "random coil" state with parallel and antiparallel orientation each, and two "fibril-like" dimer models with parallel and anti-parallel orientations.

Discussion: This project shows for a first time that a conformational change of one monomer within the dimer impedes primary nucleation, while less fluctuations and relatively large number of interactions in nucleation domains induce the nucleation to produce toxic stable species (Figure 1). Overall, the study exhibits a diverse of primary nucleation in each dimer state, suggesting distinct molecular mechanisms of fibril formation.

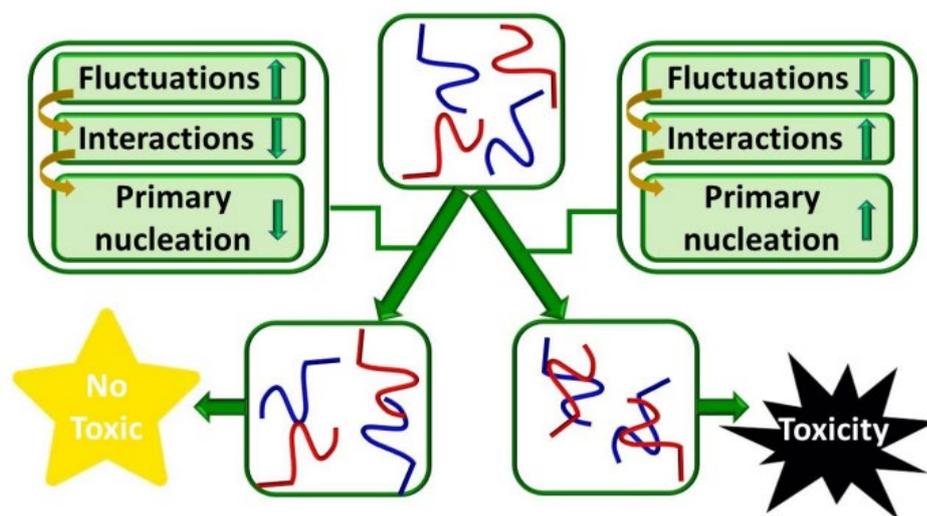


Figure 1: Proposed mechanisms that lead to toxicity due to a primary nucleation of A β dimer.

Reference:

Press-Sandler O., Miller Y. *ACS Chem. Neuro.* **2019**, DOI: 10.1021/acchemneuro.9b00437. In press.

Dual Action of Nanodiscs on Amyloid Aggregation

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Background: Protein aggregation and amyloid formation are associated with many neuropathic and non-neuropathic diseases, including Alzheimer's disease (AD) and type-2 diabetes (T2D). Human islet-amyloid polypeptide (hIAPP) and β -amyloid peptides are a histopathological hallmark of T2D and AD, respectively.¹ While, the underlying mechanisms are unclear, increasing evidence suggests that (i) the biological membrane plays a central role in catalyzing amyloid aggregation and severity of pathological conditions,² and (ii) the amyloid intermediates are toxic and are potential therapeutic targets.³ The use of nanodiscs in studying membrane protein and membrane interacting peptide/protein is emerging as a powerful tool, but are less explored in the investigation of amyloids.⁴

Aim: This study aims to overcome the key challenges posed by the cell membrane in the field of amyloid diseases using nanodiscs. First, by varying the lipid composition in the nanodisc, we intended to explore the role of membrane in catalyzing hIAPP and β -amyloid aggregation. Second, through selective optimization of nanodisc's chemical and physical properties including the encapsulated belt, we aim to control amyloid aggregation and fiber formation.

Methods: To achieve the goals of this study, we employed an integrated experimental and computational biophysical approach. Using biophysical methods including fluorescence spectroscopy, circular dichroism, NMR, high-speed AFM, calorimetry, and TEM, the role of lipids in catalyzing amyloid aggregation in the absence and presence of nanodiscs is investigated. Nanodiscs are designed using either a short-peptide (~2.2 kDa) derived from Apo A-1 (4F)⁵ or a synthetic amphiphilic polymer (PMAQA⁺) of size ~4.7 kDa.⁶ The nanodiscs were purified using size-exclusion chromatography methods and characterized using dynamic light-scattering.^{5,7} NMR experiments were performed to obtain atomistic details of β -amyloid intermediates bound to lipid-nanodiscs. *In vitro* assay was performed on human neuroblastoma cells to quantify the pathological phenotype of the nanodisc bound β -amyloid intermediates. Structural and mechanistic details of β -amyloid interaction with nanodiscs were studied using microseconds long coarse-grained molecular dynamics (MD) simulations. Parameterization of synthetic polymers in the framework of *Martini*-force-field⁸ was developed. Self-assembled peptide- or polymer-nanodiscs were built using multi-microseconds MD simulations and used to gain mechanistic insights into amyloid and nanodisc interactions.⁹

Results and Discussion: Our results show the ability of polymer nanodiscs to isolate intermediates of β -amyloid (1-40) and also to remodel fibers to pathological distinct phenotypes. Electron microscopy combined with NMR and microsecond scale MD simulation characterizes the intermediates of β -amyloid trapped by polymer-nanodiscs or remodeled from fibers at a molecular level.¹⁰ Neurotoxicity measurement of β -amyloid (1-40) monomers or oligomers treated with nanodisc depicted a substantial reduction in the A β 40 toxicity to SH-SY5Y neuroblastoma cells.¹⁰ We also show agonist and antagonist activity of the lipid-dissociated copolymers on β -amyloid (1-40) and hIAPP fibrillation, respectively, at sub-micromolar concentration.¹¹ By varying the mole percentage of the lipid compositions in the nanodiscs and size of the nanodisc, we were able to modulate the aggregation kinetics and pathological phenotypes of β -amyloid (1-40). Further, our comparative study using peptide and polymer nanodiscs of similar size (~10 nm)

containing identical lipids demonstrates the bimodal application of lipid-nanodiscs in studying amyloid aggregation pathways and controlling its amyloid aggregation.

We have also examined the effects of peptide and polymer-nanodiscs on hIAPP aggregation. Our results show that apolipoprotein-A-I mimetic (4F) peptide-encapsulated-nanodiscs (~10 nm) provides real-time monitoring of membrane-dependent hIAPP interaction and aggregation. In contrast, the PMAQA nanodiscs (~10 nm) showed the opposite action. 4F-nanodiscs substantially accelerate hIAPP aggregation, whereas PMAQA nanodiscs inhibit hIAPP aggregation. An interesting discovery is that the lipid composition and concentration could be tuned to control hIAPP's aggregation kinetics only in PMAQA nanodiscs. High-speed AFM was used to monitor the inhibition of hIAPP aggregation in real-time by PMAQA-nanodiscs and an increase in nanodisc size was observed only for 4F-nanodiscs. Heteronuclear NMR experiments show a fast and slow dynamics for the interaction of hIAPP with 4F-nanodiscs and PMAQA-nanodiscs, respectively, indicating its selective lipid and nanodisc-belt interactions. Further, multi-microseconds MD simulation provided mechanistic insights into the hIAPP's interaction with the lipid-surface in 4F-nanodiscs, and polymer belt in PMAQA nanodiscs.

Conclusion: Results from this study successfully demonstrate the dual-action of nanodiscs on probing amyloid intermediate structure and membrane interaction. Through a control and selective optimization of nanodisc chemical properties, we are able to control amyloid aggregation and the formation of toxic intermediates. Findings from this study also suggest that nanodiscs could also be used to aid in the design of new multifunctional materials for future studies of amyloid-related diseases.

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CONSERVED NDR KINASE CONTROLS RNP GRANULE ASSEMBLY TO REGULATE CELL GROWTH AND CHRONOLOGICAL LIFESPAN

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Abstract:

Aims/Purpose Adaptation to the nutritional environment is critical for all cells. RAS GTPase is a highly conserved GTP binding protein with crucial functions for cell growth and differentiation in response to environmental conditions. Inappropriate activation of human RAS GTPase has a causal role in cancer, congenital malformations, and neurodevelopmental disorders. We previously found that the conserved NDR kinase Orb6 regulates the mRNA and protein levels of Ras1 guanine nucleotide exchange factor (GEF) Efc25, by controlling the coalescence of the mRNA-binding protein Sts5 into ribonucleoprotein (RNP) granules. However, it is still unclear whether Orb6-Sts5 axis regulates RAS GTPase activity in response to the nutritional environment. Further, the molecular mechanisms by which Orb6 kinase controls Sts5 RNP coalescence are not characterized.

Methods Fission yeast *Schizosaccharomyces pombe* serves as a powerful model organism to study signaling pathways regulating nutrient sensing, cell growth, and morphogenesis due to its well-defined cell shape and growth pattern under nutrient variations. Using a phospho-specific antibody and an analog-sensitive *orb6-as2* strain, we can quantify the Orb6 kinase activity and the effect of Orb6 kinase inhibition.

Results Here, we describe a novel mechanism tethering RAS GTPase to nutrient availability in fission yeast. The conserved NDR kinase Orb6 responds to nutritional cues and regulates Ras1 GTPase activity. Under nutrient-rich condition, Orb6 kinase increases the protein levels of a Ras1 GTPase activator, the guanine nucleotide exchange factor Efc25, by phosphorylating Serine 86 of Sts5, a protein bound to *efc25* mRNA. Under nutrient-depleted condition, Orb6 kinase activity decreases, leading to Sts5 RNP coalescence and decrease in Ras1 activity. By manipulating the extent of Orb6-mediated Sts5 coalescence into RNP granules, we can modulate Efc25 protein levels, Ras1 GTPase activity and as a result, cell growth and cell survival.

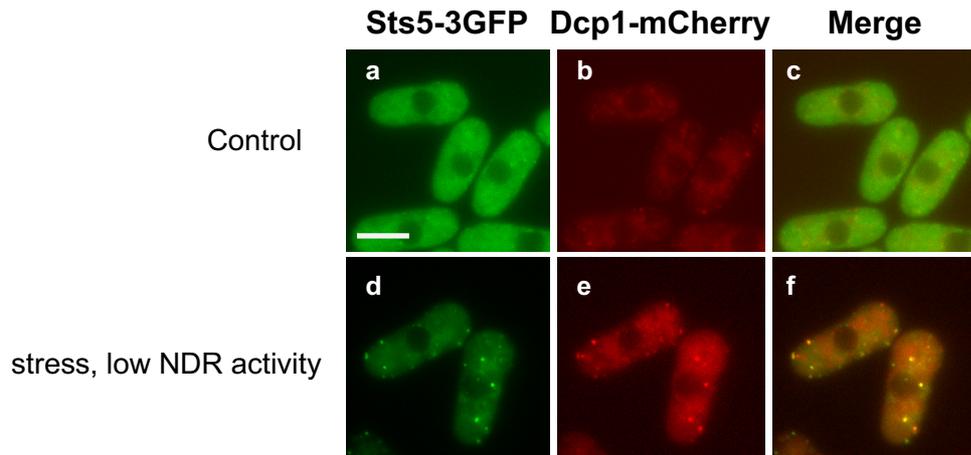
Conclusion The Orb6-Sts5-Ras1 regulatory axis plays a crucial role in promoting cell adaptation, balancing the opposing demands of promoting cell growth or extending chronological lifespan. Our findings highlight the conserved NDR kinase as a crucial enzyme in modulating cell growth and cell survival under favorable or adverse conditions, and in regulating cell adaptation and general stress response.

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Development of Nontoxic Carbon Dots and Their Applications in the Amyloid Inhibition

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Abstract: Alzheimer's disease (AD) is a leading cause of death worldwide, where no current cure, except for a therapy for delaying its onset, is available.¹ The precise mechanism of AD pathogenicity remains to be established, but one considerable molecular etiology that underlies AD involves the aggregation of β -amyloid ($A\beta$) that is produced from sequential proteolytic cleavage of amyloid precursor protein (APP) by β - and γ -secretases.² The resulting $A\beta$ can assemble into oligomers and then induce other $A\beta$ peptides to follow this misfolded process to further promote the formation of $A\beta$ fibrils in an effective "catalytic" cycle, which will eventually result in AD.³

Aims/Purpose: To redirect this "inaccurate" encoded pathway, antiamyloidogenic agents for AD have to be designed, which involves inhibiting the energetically driven assembly process of amyloid plaques by their binding with $A\beta$ monomers. To achieve this aim, many peptides,⁴ organic compounds,⁵ and conjugated nanoparticles⁶ have been examined with the aim of developing novel amyloid inhibitors. However, in addition to inhibiting $A\beta$ fibril assembly, very few of these addressed other challenges which arise with AD treatments, such as the capability to: (1) target upstream APP or secretases to cut off the $A\beta$ fibril generation resources, and (2) penetrate the blood-brain barrier (BBB), for delivery to pathological tissues. Therefore, before the development of a potential drug for better AD treatment can be realized, there is strong motivation for obtaining a novel antiamyloidogenic agent that can resolve these concerns.

Methods: Carbon dots (CDs) have recently emerged as a benign zero-dimensional nanomaterial with unique optical properties and biocompatibility.⁷ Also, CDs possess large surface to volume ratios, and this low dimensionality nanomaterial is expected to redirect the partially unfolded $A\beta$ by increasing the fibril steric hindrance as a result of their interaction with $A\beta$ monomers.

Previously, in our research group, human transferrin (HT) has been utilized to help one type of CDs (B-CDs) transport across the BBB.⁸ B-CDs have also been observed to potently inhibit the fibrillation of human insulin, a common amyloidosis model.⁹ Inspired by this, B-CDs were used as a potential BBB-permeable antiamyloidogenic agent candidate for AD treatment.¹⁰ In addition, recently, we have also successfully developed one type of CDs (Y-CDs) with excellent amphiphilicity. Due to the amphiphilicity, Y-CDs are expected to have a better BBB and cell membrane penetration capability.

To evaluate the cytotoxicity of B-CDs and Y-CDs in the cellular environment, we varied in diverse cell lines including pediatric brain glioblastoma (GBM) (CHLA-200, Daoy, CHLA-266 and SJGBM2), normal kidney (HEK293) and Chinese hamster ovary (CHO) cells lines.¹¹

To examine the inhibition activity of B-CDs against $A\beta$ fibrillation, different concentrations of B-CDs were prepared for the thioflavin T (ThT) binding assay, where ThT can specifically recognize the β structure inside the peptide to obtain a locked conformation and exhibit enhanced fluorescence upon binding to amyloid fibrils so as to alter the fluorescent spectrum with the growth of fibrils.¹⁰ However, different from B-CDs, instead of inhibiting $A\beta$ fibrillation, Y-CDs at different concentrations were investigated inside the aforementioned CHO cells that overexpress APP to observe the inhibitory effect on the production of APP and secretion of $A\beta$ to eradicate the AD cause in the beginning.

In order to study the capability of both CDs to cross the BBB, zebrafish were used as an *in vivo* model considering several advantages: 1) zebrafish don't require large space and they are cost-effective to keep;⁸ 2) adult zebrafish breed approximately every 10 days and can lay 50 to 300 eggs at a time;¹² 3) zebrafish embryos and larvae are nearly transparent, which allows researchers to observe the real-time development of tissues and any fluorescently labeled activity in zebrafish body.¹³ To investigate whether B-CDs or Y-CDs could cross the BBB, we had to intravascularly inject B-CDs or Y-CDs into the heart of anesthetized wildtype zebrafish (number: 6) at five days post-fertilization.⁸ The injected zebrafish were then observed under the confocal microscope excited with their respective maximum excitation wavelengths.⁸

Results: With four different pediatric brain GBM cell lines, B-CDs are confirmed with nontoxicity. Meanwhile, the nontoxicity of Y-CDs has been confirmed by four different cell lines (pediatric brain GBM: CHLA-200 and SJGBM2, normal kidney: HEK293, and CHO cell lines) even at a significantly high concentration as 10 μ M.

When B-CDs were introduced at 0 h incubation, it illustrated that (1) the suppression of A β 42 could be observed with only 2 $\mu\text{g mL}^{-1}$ B-CDs, and (2) the fluorescence intensity decreased when the concentration of B-CDs was increased to saturation. In addition, it was observed that adding 10 $\mu\text{g mL}^{-1}$ B-CDs after 1 h incubation, promoted a faster A β 42 aggregation than that in the presence of 10 $\mu\text{g mL}^{-1}$ B-CDs before incubation, which strongly indicates that B-CDs interact with A β 42 monomers to redirect fibril assembly at an early stage, which was supported by the ThT profile of A β 40 and theoretical data. Meanwhile, Y-CDs have revealed the ability to inhibit APP production in CHO cells which was assessed by an immunofluorescence analysis. In untreated cells there was a robust aggregation of APP which clearly indicated that these cells stably expressed human APP751. Upon treatment with Y-CDs, the expression of human APP751 was dose-dependently inhibited with the increasing concentration of Y-CDs. A similar trend was also observed when the secretory A β levels were analyzed. Compared to the culture media from untreated cells there was a ~20% reduction in the secreted A β levels following the treatment with Y-CDs at 10 μM concentration.

B-CDs alone could not cross the BBB. Therefore, in order to cross the BBB in the zebrafish model, B-CDs were conjugated with HT. With the help of HT, B-CDs eventually crossed the BBB via a receptor-mediated endocytosis and a red fluorescence was observed in the central canal of spinal cord under the excitation of 561 nm using a confocal microscope. Significantly, due to the amphiphilicity, Y-CDs alone showed the capability to cross the BBB, which was indicated by the yellow fluorescence shown in the central canal of spinal cord under the excitation of 405 nm. Additionally, Y-CDs were able to permeate into zebrafish when the zebrafish was soaked in Y-CDs aqueous dispersion, which demonstrated that Y-CDs overcame the BBB via passive diffusion.

Discussion and/or Conclusion: Both CDs are nontoxic. The excellent inhibitory effect of B-CDs on A β fibrillations was verified using a ThT assay while Y-CDs significantly inhibited the production of APP and the secretion of A β . Given the tunable properties of B-CDs, HT was engineered onto their surface for delivery and crossing the BBB in a zebrafish model. Importantly, amphiphilic Y-CDs alone also showed the ability to cross the BBB via passive diffusion using zebrafish as an animal model. Above all, both CDs were examined for cytotoxicity in different cell lines, which proved that both CDs are nontoxic. Thus, both CDs presented in this work display an excellent potential for application in the pharmaceutical industry or other areas for the treatment of AD.

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Isoform-specific protection of NMNAT against Tau-induced neurodegeneration by suppressing pathological pTau aggregation

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Purpose: Tauopathies are among the most common proteinopathies characterized by the presence of phosphorylated Tau (pTau) that forms pair-helical filaments in the brain. Molecular chaperones are involved in regulating the pathological aggregation of pTau and modulating disease progression. Here, we characterized the cytotoxicity of pTau *in vivo* using a *Drosophila* tauopathy model and investigated the neuroprotective effect of nicotinamide mononucleotide adenylyltransferase (Nmnat), an evolutionarily conserved nicotinamide adenine dinucleotide (NAD⁺) synthase with chaperone activity. Specifically, we set out to test the neuroprotective capacities of two Nmnat isoforms, a nuclear isoform Nmnat^{PC} and a cytoplasmic isoform Nmnat^{PD}, produced by stress-regulated alternative splicing ([1](#), [2](#)) and dissected the underlying protective mechanisms against pTau-induced synaptopathy.

Methods: We applied Thioflavin T fluorescence kinetic assay and transmission electron microscopy to monitor the influences of Nmnat^{PC} and Nmnat^{PD} on pTau amyloid aggregation *in vitro*. To measure the protective capacities of Nmnat^{PC} and Nmnat^{PD} *in vivo*, we established a *Drosophila* model of tauopathy using a pan-neuronal driver *elav-GAL4* to express human Tau with Nmnat^{PC} or Nmnat^{PD}. We characterized Tau-induced age-dependent neurodegenerative phenotypes by a negative geotaxis assay and measured pTau aggregation and apoptotic level in the brain through confocal imaging and biochemical analysis. To investigate the protective role of Nmnat in pTau-induced synaptopathy, we took advantage of the *Drosophila* visual system by expressing Tau and Nmnat in photoreceptors using a photoreceptor-specific driver *GMR-GAL4*. We examined the morphology and localization of mitochondria using a fluorescent reporter mito-GFP, the integrity of the synaptic cytoskeleton using a reporter LifeAct-GFP, as well as the synaptic structures by probing for Bruchpilot (Brp), a synaptic active zone cytomatrix protein.

Results: We showed that both Nmnat^{PC} and Nmnat^{PD} exhibited robust chaperone activities against the amyloid aggregation of pTau₂₃ and pK19 in a dose-dependent manner *in vitro*. In *Drosophila* models of tauopathy, endogenous Nmnat^{PC} and Nmnat^{PD} were recruited to pTau-induced vacuoles in the brain, yet showed distinct protective capacities. Neuronal expression of Nmnat^{PD} partially rescued Tau-induced locomotor defects, suppressed brain apoptosis, and reduced filamentous pTau accumulation, while neuronal expression of Nmnat^{PC} showed minimal protection (**Figure 1**). In addition, we showed that pTau aggregation impaired synaptic functions via (1) impairing mitochondrial dynamics and localization, (2) disrupting synaptic active zone integrity, and (3) stimulating F-actin accumulation that restricts synaptic

vesicle mobility and release. Overexpression of Nmnat^{PD} significantly suppressed pTau aggregation and protected against pTau-induced synaptic dysfunction by restoring mitochondria and Brp localization at synaptic terminals, and alleviating pathological F-actin accumulation.

Conclusion: Our study provides mechanistic insights of pTau-induced neurodegeneration *in vivo*, and uncovers the isoform-specific protection of Nmnat in tauopathy.

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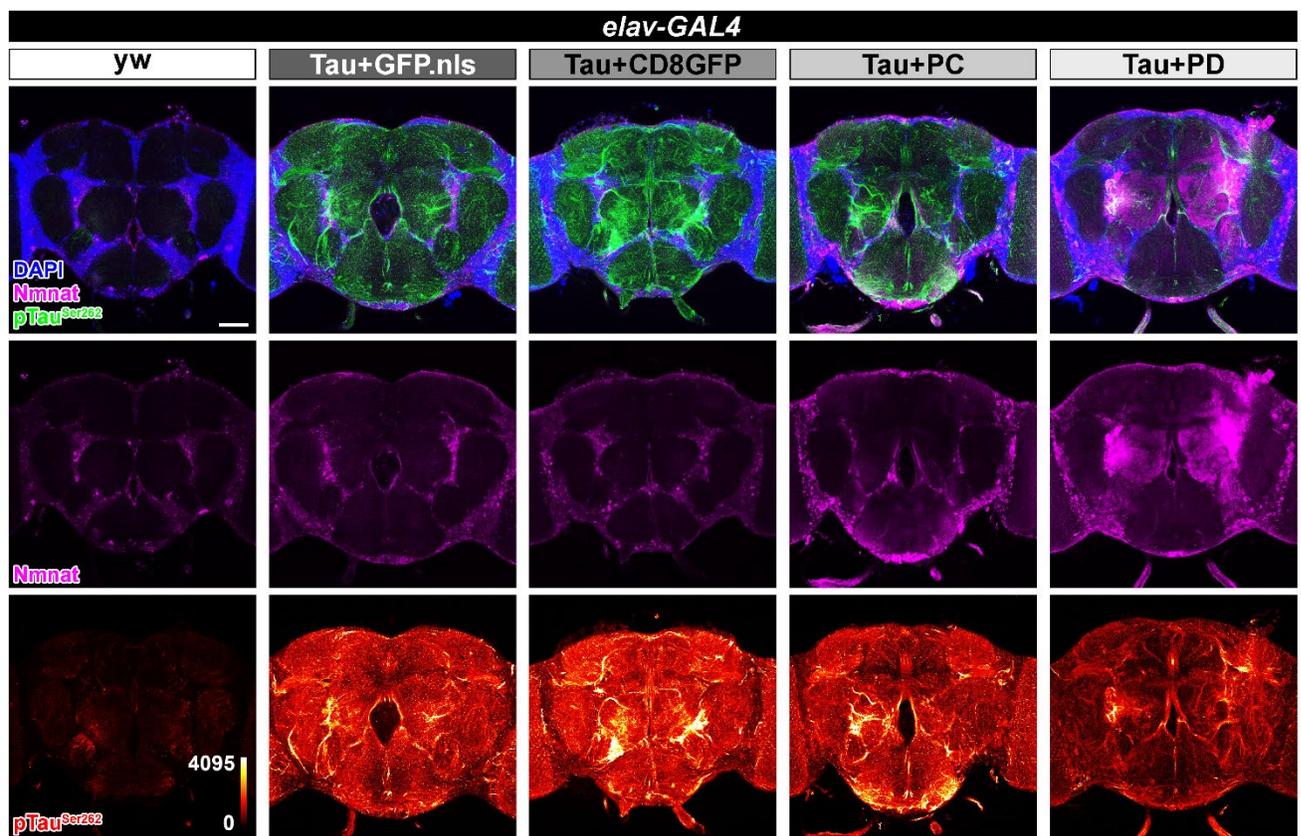


Figure 1. Nmnat^{PD} reduces filamentous pTau^{Ser262} accumulation in the brain.

Adult *yw* fly brains or fly brains with neuronal expression of Tau^{R406W}+GFP.nls, Tau^{R406W}+CD8GFP, Tau^{R406W}+Nmnat^{PC}, or Tau^{R406W}+Nmnat^{PD} by *elav-GAL4* at 20 days after eclosion (DAE) were probed for Nmnat (magenta) and pTau^{Ser262} (green) and stained for DAPI (blue). In the bottom row, the intensity of pTau is indicated by a heat map. Scale bar: 50 μ m.

Cryo-EM structures of fibrils from systemic amyloidosis

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Background:

Systemic amyloidosis is a group of protein misfolding disease, in which the production site of the fibril precursor protein does not necessarily correspond to the deposition site of the amyloid within the body. The fibril precursor protein in systemic AA amyloidosis is serum amyloid A. This protein is produced mainly in liver but deposits in spleen, kidney and other organs. In systemic AL amyloidosis, the fibril precursor is an immunoglobulin light chain. Systemic AA amyloidosis affects humans and many animal species and represents one of the best cases of a non-cerebral prion-like disorder in mammals.

Methods

We have used cryo electron microscopy to determine the molecular structures of amyloid fibrils purified from different diseases in humans and animals.

Results:

Our research provides insights into the mechanisms of protein misfolding in vivo and into the role of prion-like seeding or the molecular basis of a species barrier.

Are functional and pathological forms of alpha-synuclein so different?

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Abstract:

Aims. α -synuclein (α S) is an intrinsically disordered protein whose aggregation is strongly connected with neurodegenerative disorders including Parkinson's disease (PD), Parkinson's disease with dementia, dementia with Lewy bodies and multiple system amyotrophy. The physiological function of α S is still unclear, however, several evidences indicate that this protein may play a role in the homeostasis of synaptic vesicles (SV) during neurotransmitter release, a task that is associated with its ability to bind SV and promote vesicle-vesicle interactions. A key element for the biological properties of α S is the interaction with membranes. The equilibrium between cytosolic and membrane-bound states is indeed crucial for the physiological properties of α S, the toxicity of α S aggregates, and the kinetics of α S aggregation.

Methods. We have started a longterm research programme to tackle the main mechanisms of membrane interaction by α S monomers or aggregated species, using a variety of biophysical and biochemical techniques, including nuclear magnetic resonance, super resolution microscopy, cryo electron microscopy, FRET and cellular biophysics.

Results. Our data indicate that three distinct regions in the monomeric state of α S interact with specific binding modes with lipid bilayers [1]. These include the N-terminal 25 residues, acting as a membrane anchor, a central 'sensor' region (residues 26 to 98), modulating the overall affinity of the protein for lipid bilayers as well as the mechanism by which α S promotes the clustering of synaptic vesicles [2], and the Ca^{2+} -sensitive [3] C-terminal residues (99 to 140) remaining largely unstructured and unbound to the membrane surface.

When studying the underlying mechanisms of neuronal toxicity by α S oligomers, we found that the N-terminal anchor is also employed by these aggregates to bind biological membranes, a step that is followed by the insertion of a fibrillar core of the oligomers into the lipid bilayer resulting in the disruption of the membrane integrity [4,5].

Conclusions. These works begin to identify a link between physiological and aberrant processes involving α S. The similarities in the membrane interactions by functional monomers and toxic oligomers pose tremendous the challenges in PD research to identify therapeutic approaches that selectively target pathological aggregates without interfering with the functional form of the protein.

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Amyloidogenic nanoparticles in blood serum of patients with Alzheimer's disease

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Introduction: Biomarkers are central to current research on molecular mechanisms underlying Alzheimer's disease (AD) and other amyloid-related diseases. Further development in this field is very important for understanding the pathophysiological processes that eventually lead to disease onset. Biomarkers are also crucial for early disease detection, before clinical manifestation, and for the development of new disease modifying therapies.

Aim: The overall aim of our work was to develop a minimally invasive method for fast, ultra-sensitive detection of structurally modified peptide/protein aggregates in the peripheral blood of humans and possibly in other biological fluids. Our focus here was on developing a method to detect structured amyloidogenic oligomeric aggregates in the blood serum of apparently healthy individuals to compare it with patients in an early AD stage, and to measure the concentration and size of the aggregated amyloid nanoparticles in both cases.

Methods: Thioflavin T (ThT) is a molecule which becomes strongly fluorescent when binding to amyloid material, like amyloid fibrils formed from the Amyloid- β peptide involved in AD (1). Here ThT was added to samples of blood serum from AD patients and control individuals. We used time-resolved detection of ThT fluorescence intensity fluctuations in a sub-femtoliter observation volume element to identify ThT-active structured amyloidogenic oligomeric aggregates in the blood serum, and to measure their concentrations and sizes with single-particle sensitivity. The methodology is based on Fluorescence Correlation Spectroscopy (1).

Results: We find that both concentrations and sizes of structured amyloidogenic nanoparticles are significantly higher in the blood serum of individuals diagnosed with AD than in control subjects (2).

Conclusion: Our approach does not require immune-based probes, nor on the use of radiotracers, signal-amplification or protein separation technique. It provides a minimally invasive test for fast and cost-effective early determination of structurally modified peptides/proteins in the peripheral blood. Ongoing work aims to find out if a similar method can also be used on other biological fluids, and possibly also for other amyloid diseases than AD.

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Islet Amyloid Formation From Basic Biophysics to Next Generation Therapeutics and Studies of The Biophysical Basis Of The Cell Stress Response.

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Aims This talk will focus on the rational design of analogs of the polypeptide hormone amylin for therapeutic applications to treat diabetes. If time permits I will also touch on our recent unpublished work on modeling proteome collapse under stress and our work in applying thermodynamic integration (alchemical free energy calculations) to amyloid fiber stability.

Part-A

Background: The neuro pancreatic polypeptide hormone amylin (also known as Islet amyloid polypeptide or IAPP) is synthesized in the pancreatic beta-cells, stored together with insulin and released in response to the same stimuli that lead to insulin production. Amylin normally plays an adaptive role in metabolism, but forms pancreatic islet amyloid in type-2 diabetes (1-3). We and others have shown that rapid islet amyloid formation also contributes to the failure of islet transplants, thereby limiting this potential treatment for type-1 diabetes (4). Although not the cause of type-2 diabetes, islet amyloid formation contributes to beta-cell dysfunction and death in the disease (1-3,5). In contrast, amylin is absent in type-1 diabetes owing to the destruction of the beta cells.

Type-2 diabetes, until relatively recently, was a disease that developed later in life, typically after childbearing years. Thus, there may be little to no selective pressure to avoid islet amyloid formation. In contrast, some animals have adapted to thrive in metabolically stressful environments and here one may expect evolutionary advantageous to be provided by resistance to islet amyloid formation. For example, polar bears eat an extremely high fat diet and have a form of amylin which is less amyloidogenic than humans (6). Strikingly, mice transgenic for human amylin develop islet amyloid and diabetes if fed a high fat diet, even one with a notably lower percentage of fat than the natural polar bear diet. In addition, porcine and bovine amylin are much less amyloidogenic than human amylin and domesticated pigs and cattle are believed not to develop type-2 diabetes (4,7). Sequence differences between species have been exploited to develop first generation non-amyloidogenic analogs of human amylin for clinical applications. In particular, comparative analysis of human vs rat/mouse amylin has led to a variant of amylin, Pramlintide, which is used clinically (8). Human amylin lacks proline residues while rat/mouse amylin contains prolines at position 25, 28, and 29. Pramlintide is a triple mutant of human amylin with Ala-25, Ser-28, and Ser-29 replaced by Pro. Pramlintide is not amyloidogenic *in vitro*, except at high concentrations. Never the less, next generation bioactive analogs of human amylin with increased solubility are desired, as the low solubility of pramlintide at neutral pH precludes co-formulation with insulin formulations. However, despite considerable work, the features which control amylin amyloidogenicity are still not fully understood.

Results and Discussion: We have exploited studies of species which do not form islet amyloid *in vivo* together with *in vitro* mechanistic studies of amylin aggregation to rationally design a set of human amylin analogs which are not amyloidogenic, are not toxic to beta-cells, are much more soluble than pramlintide at physiological pH and which can be co formulated with commercial insulin preparations (6,7,9). Several of these analogs are as active as wild type human amylin to the human calcitonin receptor and to the human amylin receptor. These molecules hold considerable promise as next generation soluble amylin analogs for clinical applications.

Part-B Background: The second topic of this presentation deals with the cellular response to stress. Of the many stressors that cells encounter, thermal stress leading to heat-shock induced cell death is perhaps the best studied, but the exact mechanism is not clear. There is considerable

interest in understanding the molecular basis of cellular responses to thermal stress. Of the various biomolecules that comprise the cellular environment, heat stress has a particularly pronounced effect on proteins. The high abundance of proteins in the cell coupled with their narrow range of thermal stability and relatively low overall stability can result in dramatic changes in the proteome with only modest changes in temperature. This, combined with the role of proteins in the vast majority of critical cellular processes has led to the view that heat-shock induced cell death is the result of a loss of proteome function. However, conflicting models have been proposed. *Ex vivo* and proteome-wide thermal denaturation studies have led to the hypothesis that loss of function of a subset of key proteins results in cell death, while *in vitro* biophysical analysis of protein stability data suggest proteome collapse occurs in which a significant fraction of proteins unfold (10,11).

Results and Discussion: We resolve this conflict by developing a rigorous database of protein stability data with classification based on the source organism. Linear relationships of ΔH , ΔS and ΔC_p of unfolding with chain length and knowledge of domain size distributions in different kingdoms of life are used to parametrize our model. The model reproduces the trends of *ex vivo* experiments, predicts that heat shock results from loss of function of a subset of proteins rather than proteome collapse, and provides insight into thermophilic adaptation. The work also validates the application of *in vitro* modeling to *in vivo* analysis.

Acknowledgements: Special thanks to Professors Martin Zanni, Erwin London, Kostas Thalassinou, Stephen Kahn, Rebecca Hull, and Ann Clarke for numerous helpful discussions and for long time collaborations on other aspects of islet amyloid formation.

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Peptide and metabolite assemblies: Extension of the amyloid hypothesis Ehud Gazit

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Abstract:

The formation of amyloid fibrils is being observed with both disease-associated and disease-unrelated proteins and polypeptides, suggesting a generic phenomenon for energy-stable aggregative states of proteins [Gazit, 2002]. The ability of phenylalanine to form amyloid-like structures [Adler-Abramovich et al., 2012] and their relevance to phenylketonuria (PKU) had extended the amyloid phenomenon beyond proteins and peptides for the first time [Adler-Abramovich et al., 2012]. The phenylalanine amino acid formed the assemblies in the mM concentrations. This is consistent with the PKU pathology as the phenylalanine amino acid is accumulated due malfunction of the phenylalanine hydroxylase (PAH) enzyme and PKU is classified by the levels of blood phenylalanine. Classic PKU is defined when levels of phenylalanine are $> 1200 \mu\text{M}$. Later work had demonstrated that also other metabolites, including amino acids and nucleobases, could form amyloid-like assemblies [Shaham-Niv et al., 2015, 2017]. These building blocks were relevant to other inborn error of metabolism disorders in which other metabolites could accumulate due to the lack of specific enzymes. These building blocks form typical amyloid fibrils with the same morphology, dye-binding specificity, and electron diffraction pattern as protein amyloids [Gazit, 2016]. Interestingly, these assemblies show other properties similar to protein amyloid, including binding to membranes [Shaham-Niv et al., 2018b], inhibition by various polyphenols [Shaham-Niv et al., 2018a] and intrinsic fluorescence in the visible part of the electromagnetic spectrum [Shaham-Niv et al., 2018c]. Therefore, it appears that the metabolite amyloids not only share both the morphology and chemical–physical characteristics as protein amyloid but also their specificity toward biological targets. We had recently established a yeast model to study metabolite assembly in living organisms [Laor et al., 2019]. The ability of metabolite also to form functional assemblies [Aizen et al., 2018; Makam et al., 2019] is also interesting due to the ability of protein and peptide amyloids to form functional assemblies [Cherny and Gazit, 2008; Bera et al., 2019].

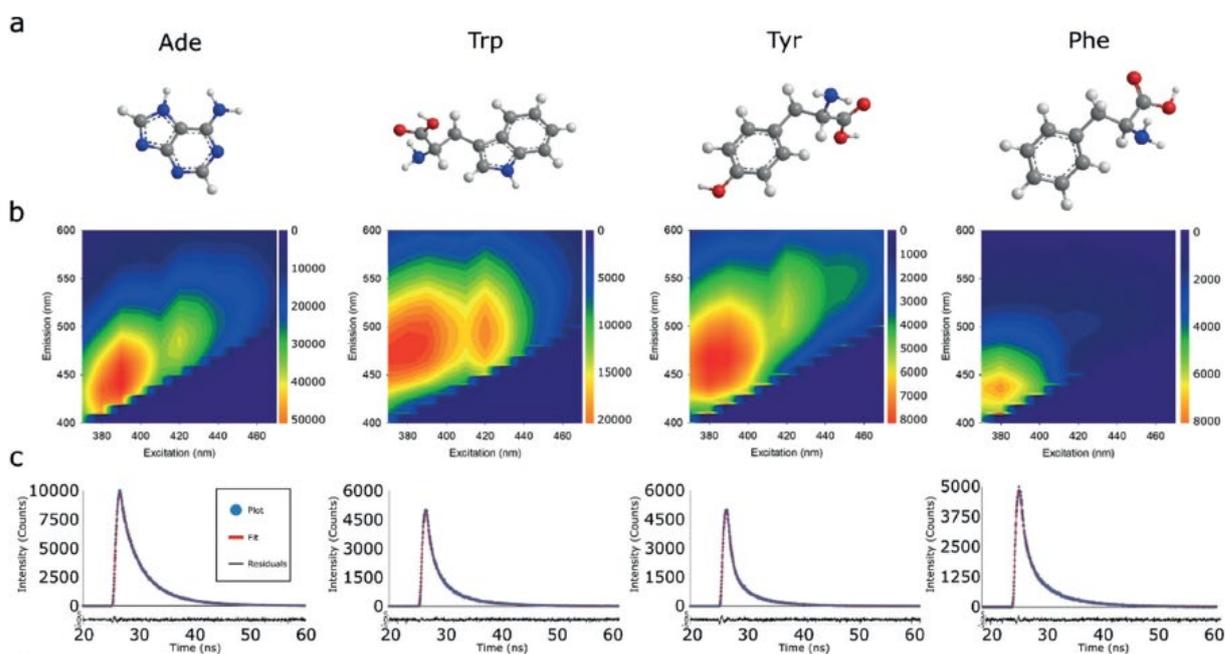


Figure 1: The ability of metabolite amyloid-like assemblies to form fluorescent architectures [taken from Shaham-Niv et al, 2018c].

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Islet amyloid polypeptide cytotoxicity directly correlates with amyloidogenicity

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Abstract

Aims/Purpose: Pancreatic islet amyloidosis by the hormone IAPP is toxic to insulin-producing beta-cells and contributes to diabetes and islet transplantation failure, but the mechanism(s) of IAPP amyloidosis are not known. In previous papers, we defined the nature of toxic IAPP intermediates (1) and identified a new cellular mechanism of IAPP-induced beta cell death (2, 3). Here we critically test the hypothesis that toxicity correlates with the lag phase length of amyloid formation, by determining whether altering the length of the lag phase affects the duration of toxicity.

Methods: We carried out concurrent biophysical and beta-cell biology studies in order to characterize the different species that form during human IAPP (h-IAPP) amyloid formation, and simultaneously assess their effects on cultured rat INS-1 beta-cells. We examine the rate of amyloid formation, and the onset and duration of toxicity of wild-type and mutant IAPP peptides under a range of conditions that yield rates of amyloid formation that differ by over 450-fold. In total, we conducted 22 distinct measurements and plotted the lag phase length of each peptide condition versus their respective duration of cytotoxicity, to determine whether there is a relationship between amyloidogenicity and cytotoxicity. Alamar Blue reduction assays and light microscopy were used to measure β -cell metabolic function and morphology, while thioflavin-T binding experiments and transmission electron microscopy (TEM) were used to follow amyloid formation.

Results: The rate of h-IAPP amyloid formation is both concentration- and temperature-dependent. Lowering the temperature increases the length of the lag phase as does lowering the concentration of h-IAPP, while increasing the concentration or temperature shortens the lag phase. Our concentration- and temperature-dependent data unequivocally show that cytotoxicity is linked to pre-amyloid intermediates in the lag phase of amyloid formation, and provide compelling evidence for a correlation between lag phase length and duration of toxicity. Amyloid fibrils formed by wild-type and mutant peptides are not toxic. We also employed a set of rationally designed mutants which either decrease or increase the rate of amyloid formation compared to h-IAPP (4-7), to extend the dynamic range of our experiments and further test the potential relationship between duration of lag time and toxicity. Mutants that aggregated faster (4) displayed a more rapid onset and shorter duration of toxicity relative to h-IAPP, while the slower aggregating mutants (5) shifted the onset and peak in toxicity to later time points, and increased its

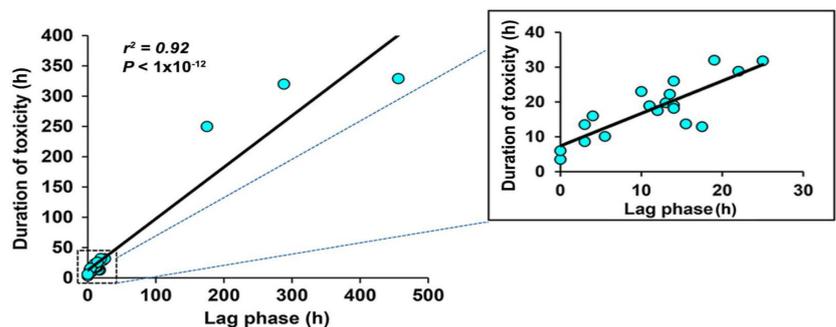


Figure 1. There is a highly statistically significant linear correlation between the lag phase length of amyloid formation and the duration of toxicity. Linear regression analysis of concurrent thioflavin-T binding assays and Alamar Blue metabolic assays were carried out for the 22 independent experiments. Inset shows the data at early time points. The measured lag phase lengths spanned a range from less than 1 hour to more than 450 hours. Data indicate a highly statistically-significant linear relationship between the length of the lag phase and the duration of toxicity ($r^2=0.92$, $P<1 \times 10^{-12}$).

duration. We also analyzed a mixture of h-IAPP and a h-IAPP point mutant that acts as an inhibitor of amyloid formation by wild-type h-IAPP in the sense that it prolongs its lag phase (6, 7). A 1:1 molar mixture of h-IAPP and the inhibitor has a lag phase that is approximately twice as long as that of h-IAPP alone, and exhibits a similar increase in the duration of toxicity. Together, the data show a striking linear correlation ($R^2=0.92$, $P<1\times 10^{-12}$) between the length of the lag phase of amyloid formation and the duration of toxicity towards rat INS-1 beta-cells over a >450-fold variation in the lag phase length (Figure 1).

Conclusion: The data demonstrate a direct relationship between cytotoxicity and amyloidogenicity, and provide strong compelling evidence that IAPP toxic species are pre-fibrillar. The results provide a method for predicting when the toxic intermediates form during *in vitro* studies, facilitating their isolation. The findings indicate that pre-amyloid species are critical targets for therapies aiming to alleviate IAPP-mediated cytotoxicity.

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How do the intrinsic and extrinsic factors modulate the amyloid aggregation of human islet amyloid polypeptide?

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Aims and purpose:

The aggregation of misfolded proteins or peptides into insoluble amyloid fibrils is a characteristic of more than 20 misfolding diseases, such as Alzheimer's disease, Parkinson's disease and type 2 diabetes mellitus (T2D). Amyloid is a generic term for a protein aggregation state in which the proteins bind to each other in a β -sheet conformation and forms fibrils alone or in interaction with membranes. In T2D, the islet amyloid polypeptide (IAPP), a 37 amino acids peptide synthesized by the pancreatic islet β -cell and co-secreted with insulin, forms amyloid fibrils which induce β -cells apoptosis, leading to a decrease in the pancreatic β -cell mass.

The composition of the pancreatic β -cell granules is extremely complex and contains many components that influence hIAPP fibril formation and hIAPP toxicity. Here, we investigate the influence of the flanking peptides, of insulin and zinc all present in the β -cell granules on hIAPP fibril formation as well as hIAPP-induced membrane damage.

Methods:

Using a pluridisciplinary approach conjugating peptide chemistry, biophysics and biochemistry, we have studied hIAPP fibril formation, hIAPP-induced membrane leakage and toxicity in the absence and presence of the flanking peptides, insulin and zinc. In addition, to gain more insight into the importance of the residue histidine 18, we have synthesized four analogues where histidine has been replaced by arginine (H18R-IAPP), lysine (H18K-IAPP), glutamic acid (H18E-IAPP) and alanine (H18A-IAPP) and we have performed the same biophysical and biological study.

Results and discussions:

Our results showed that the flanking peptides slightly inhibit hIAPP-fibril formation and hIAPP-membrane damage, but do not modulate hIAPP-induced toxicity.

However, insulin, even at a low ratio, inhibit importantly fibril formation of hIAPP and its mutants. In addition, insulin modify the kinetics of hIAPP-membrane leakage for both the wild type and the mutated peptides. To explain the differences observed, we propose a model of interaction.

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Can inhibitors of IAPP aggregation act via a different mechanism depending on whether or not membranes are present?

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Abstract:

Aim: Type 2 diabetes (T2DM) is associated with aggregation of the human islet amyloid polypeptide (hIAPP) into cytotoxic amyloid species. Inhibition of hIAPP amyloid formation is considered to be an effective strategy to help combat T2DM. Recently, a family of di-phenyl pyrazole (DPP) amyloid inhibitors has been developed that act as promising oligomer modulators to combat amyloid diseases [1-3]. For none of the DPP compounds, the effect on hIAPP has been tested. Yet hIAPP is an interesting target, because it forms oligomers that have some features that are different from those of other well-studied amyloid forming proteins [4]. Here we set out to elucidate whether DPP compounds can be effective against aggregation of hIAPP. To study this, we selected the DPP-derived small-molecule inhibitor anle145c, because it structurally resembles the very lipophilic lead compound anle138b and is likewise an efficient inhibitor of amyloid aggregation, but it has the advantage of being more water soluble [1] and therefore more amenable to biophysical investigations.

In addition, we aimed to investigate the effect of the inhibitor on hIAPP aggregation in the presence of lipid bilayers. The reason for this is that membranes are a potential target for toxicity of hIAPP *in vivo*, e.g. by inducing membrane damage and leakage, and because interaction of hIAPP with membranes can affect the aggregation process in different ways.

Methods: The methods used to study cytotoxicity and aggregation in solution are described in [5]. Briefly, cytotoxicity of hIAPP in the absence and presence of anle145c was studied using insulinoma (INS-1E) cells. Aggregation was followed by ThT fluorescence, ¹H NMR spectroscopy, electron microscopy, dynamic light scattering and atomic force microscopy. Changes in secondary structure were followed using circular dichroism. Lipid bilayers were prepared from mixtures of phosphatidylcholine and phosphatidylserine and permeability properties were assayed by calcein leakage experiments as described [6].

Results: We demonstrate that incubation of hIAPP with the inhibitor yields ~10 nm-sized non-cytotoxic oligomers, independent of the initial aggregation state of hIAPP. We also demonstrate that the inhibitor acts in a very efficient manner, with sub-stoichiometric concentrations of anle145c being sufficient to (i) inhibit hIAPP-induced death of INS-1E cells, (ii) prevent hIAPP fibril formation in solution, and (iii) convert preformed hIAPP fibrils into non-cytotoxic oligomers.

Preliminary results showed that anle145c in the presence of membranes inhibits aggregation as well as hIAPP-induced membrane leakage. We propose a model in which anle145c traps hIAPP as non-cytotoxic oligomers in solution, but also efficiently interacts with oligomers that are present in a more hydrophobic membrane environment.

Conclusion: Our findings indicate that anle145c inhibits aggregation of hIAPP in solution at sub-stoichiometric concentrations and is able to convert mature amyloid fibrils into non-toxic anle145c-stabilized oligomers. This suggests that anle145c-stabilized oligomers form a thermodynamic sink for the preferred aggregation state of hIAPP and anle145c in solution. In the presence of membranes however, anle145c may act on different oligomeric species, thereby inhibiting membrane leakage. Comparative analysis of membrane-involving experiments with the lead compound anle138b will reveal whether both DPP compounds show a similar mechanism. In any case, the results suggest that DPP compounds are promising candidates to inhibit protein aggregation in case of T2DM and they highlight their potential as small molecule oligomer modulators for T2DM and for amyloid diseases in general.

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What is the mechanism of huntingtin misfolding? Can we develop tools to monitor and inhibit it?

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Abstract:

Aims/Purpose: The misfolding and aggregation of huntingtin is one of the hallmarks of Huntington's disease, but the molecular mechanisms of misfolding remain poorly understood. Such knowledge would be useful for the design of biomarkers and therapeutics that detect and/or prevent the formation of toxic, misfolded species. Our central goal was to structurally characterize the misfolding intermediates and to find specific conformationally specific binders that were either small molecules, peptides or proteins.

Methods: For structural characterization, we used a combination of EPR, NMR, electron microscopy and other biophysical techniques. For finding binders, we tested small molecules, obtained conformationally specific peptides via molecular evolution and raised antibodies against defined misfolding intermediates.

Results: We found that huntingtin misfolding is a stepwise process, in which early helical intermediates precede the formation of β -sheet containing oligomers and fibrils of various structures. Having isolated the different misfolded species, it also became possible to obtain conformationally specific small molecules, peptides and antibodies. Some of these binders also potently inhibited misfolding. Among the most potent misfolding inhibitors were peptides specific for fibrils. The peptides appeared to protect from seeding by blocking seeding-competent surfaces. The biological properties of these binders are currently tested in cultured cells and animal model systems of Huntington's disease.

Discussion and/or Conclusion: Our structural studies defined basic mechanisms of huntingtin misfolding and led to the generation of specific binders that are useful biomarkers. Further development may also lead to therapeutically molecules.

Electron and NMR Crystallography

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1. JEOL RESONANCE Inc., Japan 2. RIKEN-Spring8 Center and RIKEN-JEOL Collaboration Center, Japan

Abstract:

Determining the crystal structure of chemical compounds and biological molecules, by defining the precise spatial arrangements of their atoms in a crystalline state, is crucial to understanding their function and reactivity. In addition to crystalline structure, analyzing the hydrogen bonding strength between hydrogen and electronegative atoms is vital in applications such as medicine, engineering, materials science and pharmaceutical research. This is due to the influence of hydrogen bond strength on molecular function and inter- and intra-molecular packing stability. Elucidating the structure of molecular crystals at atomic resolution and defining the position of hydrogen atoms is critical to understanding their complex hydrogen-bonding networks.

Single-crystal X-ray diffraction (SCXRD) is a fundamental technique for determining the structure of large single crystals (>10-100 μm) and their hydrogen-bonding networks. However, smaller crystals rarely produce enough diffraction spots for structural elucidation. In addition, the hydrogen atoms are poorly located by SCXRD. So, although SSNMR provides chemical shift values and information on the proximity between hydrogen atoms and internuclear distance measurements ($^1\text{H-X}$ [$\text{X}=\text{}^{13}\text{C}$, ^{15}N]), its combination with XRD and quantum computation is required to gain a comprehensive understanding of crystal structures and hydrogen-bonding networks of unknown compounds.

In this combined approach, termed NMR crystallography, XRD is used to determine the position of every atom in a crystal except hydrogen. Quantum computation then places the hydrogen atoms in rational positions to be verified using SSNMR, which directly measures nitrogen and carbon atoms and assures atomic assignments.

However, elucidation of the structure and hydrogen-bonding network are still challenging for active pharmaceutical ingredients (APIs) which forms nano- and microcrystals that are too small for SCXRD. Although electron diffraction (ED) or electron 3D crystallography are quite recently proved to be able to determine the structures of such nanocrystals owing to their strong scattering power, these techniques still lead to ambiguities in the hydrogen atom positions and misassignments of atoms with similar atomic numbers such as carbon, nitrogen, and oxygen. Here, we propose a technique combining electron diffraction (ED), solid-state NMR (SSNMR), and first-principles quantum calculations to overcome these limitations.

The rotational ED method is first used to determine the positions of the non-hydrogen atoms of L-histidine as a demonstration. A set of ED patterns under the uniaxial rotation was collected (Fig 1a). ED patterns from three different crystals were merged for structural solution. The initial structure was solved by the direct method. However, carbon, nitrogen and oxygen atoms are wrongly assigned and, in addition, hydrogen positions are poorly located (Fig 1b). Finally, four candidate structures were assigned with the chemical knowledge of L-histidine and ^1H - ^{14}N correlation NMR spectra [2] (Fig 1c). Bond lengths were then determined by measuring $^1\text{H-X}$ dipolar interactions using 2D inversely proton-detected cross-polarization with variable contact time (invCP-VC), and structures were finally refined with these bond lengths using SSNMR. To identify the correct structure, gauge-including projected augmented wave (GIPAW) calculations and SSNMR were combined in the NMR crystallography workflow. GIPAW optimizes a crystal structure by relaxing the atomic positions to local minima of the energy surface in addition to providing NMR parameters, such as isotropic chemical shifts and quadrupolar couplings. The results showed the LH1 structure to be the most energetically favorable, a finding which was ratified by root-mean-square deviation (RMSD) calculations. (Fig 1d)

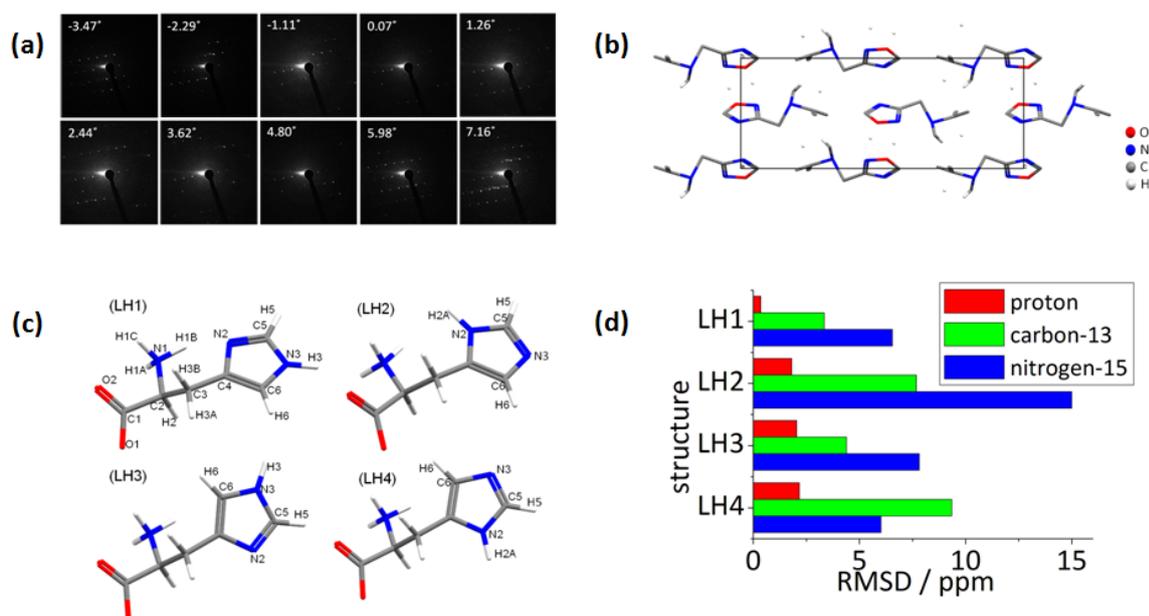


Fig 1. (a) A set of ED patterns of L-histidine at various rotation angles. (b) Initial structure solved by the direct method. (c) Four plausible candidates. (d) RMSD comparison of calculated and experimental NMR chemical shifts.

We have also successfully solved the unknown structure of cimetidine ($C_{10}H_{16}N_6S$) by the current method. Cimetidine is a histamine H_2 receptor antagonist that inhibits stomach acid production, used primarily in treating heartburn and peptic ulcers. It is known to take various crystal forms (A, B, C and D/Z) and the monohydrates (M1, M2 and M3). The needle-like structure of CB in its crystal form makes it challenging to produce a single crystal for XRD analysis, and it often crystallizes in a mixed state with crystal form C, limiting PXRD use.

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Can studying interaction of metal-ion with amyloid- β impact development of ESR spin labeling?

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Abstract: The talk will focus on the use of pulsed ESR to understand the microscopic interactions of the Cu(II) ions with amyloid- β peptide ($A\beta$). Intriguingly the ESR data shows that the Cu(II) ions coordinate largely to only two His residues at a time and that fluctuations in this coordination likely has major impact on the aggregated state of the peptide. The insight gained by these results can be exploited to design incisive spin labeling approaches for proteins. The talk will describe recent efforts to bind Cu²⁺-ions site selectively in proteins and in DNA. In proteins, the spin probe is assembled *in situ* from natural amino acid residues and a metal salt, and requires no post-expression synthetic modification. Initial results show that the resultant Cu²⁺-probe potentially *provides* distance distributions that are *five times narrower* than the common protein spin label – the approach, thus, potentially overcomes the inherent limitation of the current technology, which relies on a spin label with a highly flexible side-chain. We describe the use of this approach to resolve protein conformational dynamics, to locate native paramagnetic ions in proteins, and to resolve relative orientations of different protein segments.

References:

(Use Arial font, size 11 for all sections; Single line spacing; Figures and Tables allowed; 2 pages maximum with 1 inch top and left, 0.5 inch bottom and right margins)

Do the individual amyloid aggregates follow the prion model?

Takahiro Watanabe-Nakayama

Nano Life Science Institute, Kanazawa University, JAPAN

Abstract:

Aims/Purpose

The correlation between structural dynamics of amyloidogenic proteins and symptoms/phenotypes has been generalized among different amyloid diseases^{1,2}. We have reported that high-speed atomic force microscopy (HS-AFM) is a powerful tool for revealing structural dynamics of amyloidogenic proteins, showing direct visualization of structural dynamics of amyloid β^3 and IAPP⁴ aggregates. HS-AFM take movies at nanometer scale resolution in liquid. Thus, HS-AFM can show structure and its change at single aggregate level without purification of individual aggregate species in a course of aggregation pathway. To broaden the scope, here we report the results of HS-AFM observation of α -synuclein aggregation which leads to synucleinopathies including Parkinson's disease, dementia with lewy bodies and multiple system atrophy.

Methods

The recombinant human α -synuclein was prepared by using *E. coli* expression system. The monomeric α -synuclein was purified from the lysate by ammonium sulfate precipitation followed by a successive chromatography of anion exchange and gel filtration. The fibrils were prepared by incubation of monomeric α -synuclein with stirring. HS-AFM was operated according to ref. 5. The fibrils were mounted on the cleaved mica disc on the glass rod on the scanner. After observation individual fibrils, the fibril growth was initiated by replacement of buffer solution with the monomeric α -synuclein solution in the sample chamber. The movie analysis was performed according to ref. 6.

Results

Most of α -synuclein fibrils had ~10 nm thickness and showed straight or slight periodicity in their height profiles (Fig. 1a-b). After addition of monomeric α -synuclein, individual fibrils started to grow in the manner by which the produced fibril structure was same as the original fibril and which the growth speed was extremely different between both ends in single fibrils (Fig. 1a and c). Some fibrils were fragmented, nevertheless, the fibrils kept their growth (Fig. 1c).

Discussion

The results showed that α -synuclein fibril growth manner is consistent with the self-template replication model which is widely accepted in various amyloidogenic protein aggregation pathway. This observation system can be used for characterization of α -synuclein aggregation enhancer and inhibitor properties.

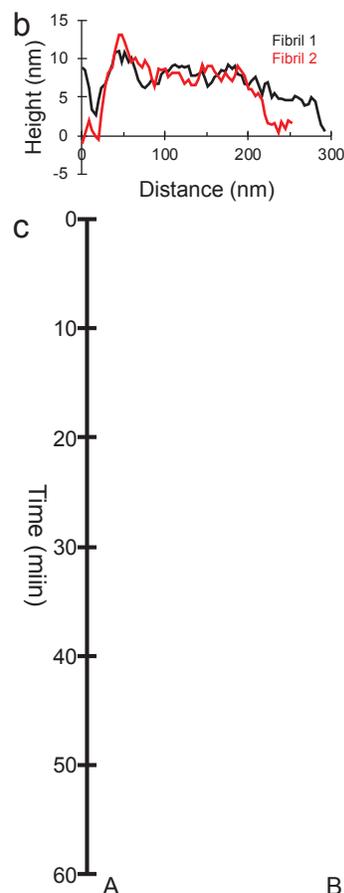


Fig. 1. HS-AFM observation of α -synuclein fibril growth.

(a) HS-AFM images of representative fibrils during their growth. (b) Height profile of the selected dashed lines 1 and 2 (A to B) in the images. (c) Kymographs of HS-AFM movies of fibril 2 and 3 in (a).

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Interaction of DPP compounds with oligomers and aggregates: implications for neurodegenerative disease and beyond

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Abstract: (Please use subsections such as Aims/Purpose, Methods, Results, Discussion and/or Conclusion)

Aims/Purpose: We develop small molecule inhibitors of aggregation which interfere/bind to oligomers with the ultimate goal to bring them in the clinic for treatment and diagnosis of neurodegeneration

Methods: We use methods from biophysics, specifically NMR but also AFM and EM to elucidate the structure of oligomers bound and unbound to the small molecule oligomer modulators derived from diphenylpyrazole (DPP) compounds and back this up with efficacy in animal models (1)

Results: Interference with the formation of oligomers can be shown for a-synuclein in vitro and in vivo as well as the efficacy in various animal models for early and late treatment of Parkinson's, Alzheimer's disease as well as multisystem atrophy and type II diabetes. Binding of such DPP compounds to oligomers in the membrane can be shown with DNP enhanced NMR. If time permits, further use of the DPP for diagnosis will be discussed.

Discussion: Interference with aggregation works very well with DPP compounds in mice and higher animals which will be discussed in the light of additional developments (2).

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Atomic Resolution Map of the Soluble Amyloid Beta Assembly Toxic Surfaces

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Abstract:

Purpose

The purpose of the talk will be on addressing the long-outstanding fundamental question of what structural attributes and interactions cause the toxicity of nanoparticles formed by peptide self-association, such as the soluble A β assemblies (A β_n) (1). A β is a prototypical amyloidogenic system that serves as a benchmark model for several other nanoparticle-forming polypeptides. However, due to their transient nature, the characterization of A β_n toxic species is challenging and remains elusive. Although it has been hypothesized that cytotoxicity arises from exposure of “toxic surfaces” shared by multiple soluble A β_n species, these exposed toxic interfaces are currently unknown.

Methods

In order to map the structural determinants of A β_n toxicity, we propose an experimental design in which we first modulate the A β_n cytotoxicity through a library of catechins and then we interrogate our catechin-modulated A β_n library at different levels of spatial resolution through an integrated set of complementary techniques (1-5). These include primarily solution NMR methods, such as ¹⁵N and ¹H dark-state exchange saturation transfer (DEST), in combination with other NMR relaxation experiments to probe A β monomer – oligomer contacts. Our NMR data were complemented by dynamic light scattering, fluorescence spectroscopy with multiple reporter fluorophores, electron microscopy and wide-angle X-ray diffraction (WAXD) in the presence of model membranes. The covariance analysis of the resulting data matrix reveals a unique cluster of molecular attributes that differentiates toxic vs. non-toxic A β assemblies. The covariance analysis relies on statistical methods such as agglomerative clustering and singular value decomposition typically employed in the analysis of microarrays for the purpose of identifying gene clusters. Here we show that when these clustering techniques are applied to matrices of combined NMR relaxation, fluorescence, DLS and WAXD data, it is possible to reliably identify previously elusive ensembles of coupled residues linked to nanoparticle toxicity. To the best of our knowledge, this is the first time that the determinants of A β_n neurotoxicity are investigated through such an integrated covariance-based experimental design.

Results

We elucidated the molecular signatures that differentiate toxic vs. nontoxic A β assemblies, including the atomic-resolution map of the previously elusive A β_n “toxic surfaces” (1). Our data reveal that toxicity is unexpectedly linked to the exposure of specific hydrophobic surfaces and their ability to interact with A β monomers and cell membranes. Whereas increased exposure of the central hydrophobic region is required for toxicity, we find that shielding of the highly charged N-terminus from A β monomer recognition enhances the toxicity of A β_n . Surprisingly, the patterns of A β assembly–monomer recognition that correlate with toxicity do not extend to C-terminal region, where the β_2 hydrophobic site is located. Instead, the β_2 region is essential for the lamination of multiple β -sheet layers required for the formation of thermodynamically stable, but non-toxic, fibrils. We also show that the toxic A β_n surfaces we mapped are critical for the binding of A β_n to lipid membranes and for forming membrane-embedded β -sheet structures, which compromise the integrity of the cell membrane. Hence, in this manuscript, we not only map the toxic

surfaces of A β_n , but we also establish the molecular mechanism underlying the toxicity of these A β_n surfaces.

Discussion

The significance of our results is three-fold (1): (a) The atomic resolution map of the A β_n “toxic surfaces” and interactions provides a general foundation to define structure-toxicity relationships of nanoparticles formed by A β and other nanoparticle-forming amyloidogenic peptides, for which A β serves as a model system; (b) We investigate A β_n in the presence of membranes, which are believed to be key toxicity determinants; (c) The proposed experimental design and analyses, as outlined above, are transferable to other nanoparticle-forming amyloidogenic peptides.

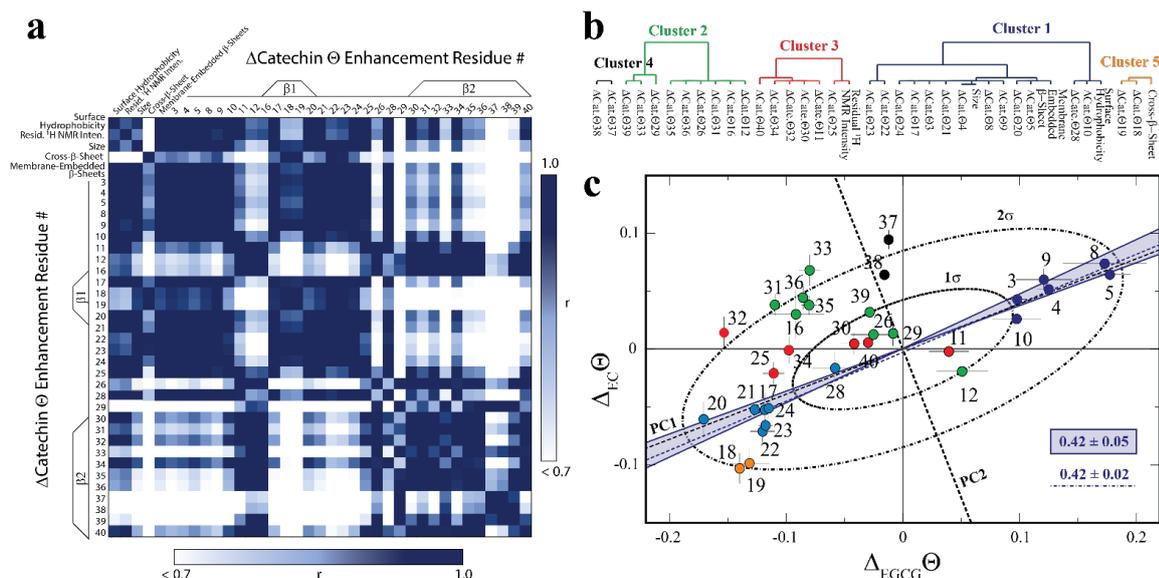


Fig. 4 – Identification of the determinants of A β assembly toxicity through agglomerative clustering and Singular Value Decomposition (SVD) (1)

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Properties of Amyloid beta (A β) Aggregates and Inhibition of A β Aggregation

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Alzheimer's disease (AD) is a neurological disorder that is characterized by the accumulation of soluble and insoluble amyloid oligomers in the brain. The major components of these oligomers are amyloid beta (A β) peptides consisting of 40-42 amino acids. It is widely acknowledged that the transition from monomeric A β to a misfolded aggregate state is related to the onset of AD. Therefore, the current potential therapeutic strategies for the treatment of this disease includes the prevention of A β aggregation using small molecules. We have employed a plethora of computational and theoretical techniques to investigate the structures and properties of the aggregated forms of A β and their interactions with a variety of small molecules. The results obtained from these studies will provide an atomic level understanding of these processes and advance our efforts to develop effective therapeutic strategies for this disease.

What secondary structure should be mimicked in peptide derivatives to inhibit the aggregation of amyloid proteins?
Helix or beta-hairpin?

Sandrine Onger
BioCIS, CNRS/Université Paris Sud
Université Paris Saclay

Aims/Purpose

At least 20 human degenerative diseases, named amyloidosis, have been currently identified and involve misfolding and misassembly into various aggregate structures of more than 30 proteins. Among these diseases, Alzheimer's disease (AD) and Type 2 diabetes (T2D) are of major concern affecting approximately 47 and 400 million people worldwide respectively. No etiological treatment exists against these two diseases which leads irremediably to the death of neuronal and pancreatic β -cells respectively. Clinical studies suggest that they are linked and that T2D might be an increased risk for AD. Aggregates of $A\beta_{1-42}$ and hIAPP amyloid proteins, involved in AD and T2D respectively, and in particular soluble oligomer species are highly suspected to be involved in neuronal and pancreatic β -cells death respectively. The strategy aiming at preventing the presence of toxic oligomers by preserving monomer species of $A\beta_{1-42}$ and hIAPP, that are described as non toxic and more easily cleared, appeared to us very appealing. β -sheets and β -strands mimics are designed to interact with β -sheets rich toxic aggregates (oligomers and fibrils). Can helix mimics also be an alternative strategy for trapping the non-toxic monomeric forms by targeting α -helix conformation before the switch to β -sheet conformation?

Methods

- i- We designed and synthesized β -hairpin and helix peptidomimetics targeting either $A\beta_{1-42}$ and/or hIAPP;
- ii- We developed new biophysical techniques to identify and separate soluble monomer and oligomer species of $A\beta_{1-42}$ and hIAPP;
- iii- We evaluated our peptidomimetics on the aggregation of $A\beta_{1-42}$ and hIAPP using these new techniques.

Results

We demonstrate that both β -hairpin and helix mimics are attractive molecules reducing efficiently the aggregation process and maintaining the presence of monomer species of amyloid proteins.

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Engineered peptides for total degradation of Amyloid Tanmay Mondal and Bhubaneswar Mandal

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Abstract:

Aim: Interneuronal deposition of misfolded Amyloid β ($A\beta$) protein aggregates is believed to be one of the causes of Alzheimer's disease (AD). Usually, site-specific cleavage of the amyloid precursor protein (APP) by α -secretases do not generate $A\beta$. Also certain proteolytic enzymes can metabolize amyloid. However, when these natural enzymes fail, stone-stable plaques form that cannot be excreted by the body system. We wanted to develop an artificial proteolytic enzyme-like machinery that would explicitly recognize the amyloid and chemically destroy them.

Methods: We have designed and developed a peptide-based construct that is intended to recognize the homologous sequence on $A\beta$ or APP by supramolecular association and cleave them at the α -secretase cleavage site by a cascade of proximity induced chemical reactions to generate non-toxic sAPP α and reduce the generation of toxic $A\beta$. Such constructs are termed as the "artificial α -secretases" (ASs, Figure 1).¹

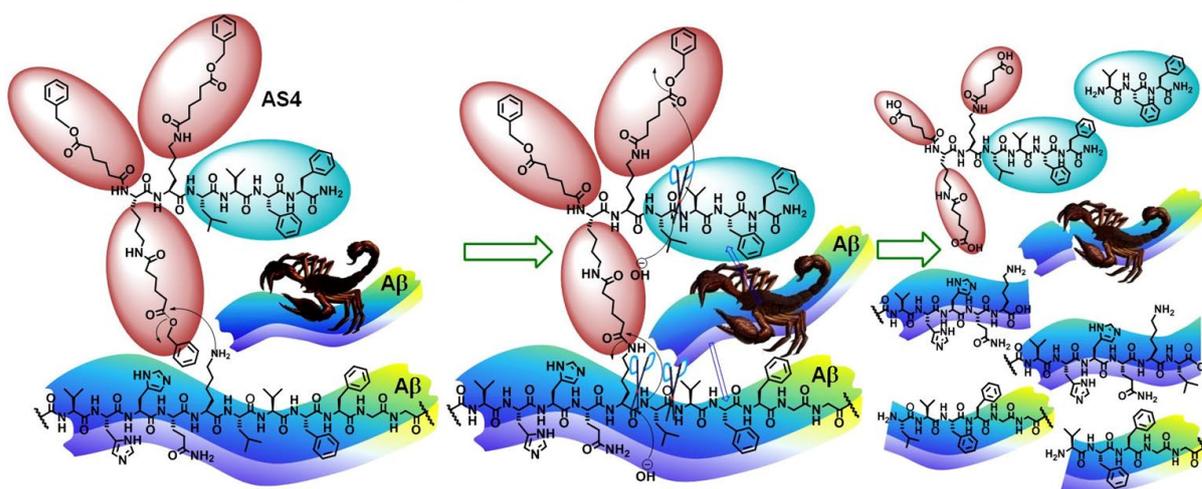


Figure 1

Results: We first confirmed the $A\beta$ -degradation potential of ASs. Plausible routes of the proteolysis of a modified $A\beta_{12-21}$ fragment and $A\beta_{1-40}$ peptide by ASs were derived from MALDI mass data analyses. Site-selective proteolysis between ¹⁶Lys-¹⁷Leu and ¹⁷Leu-¹⁸Val was noted that closely resembles the cleavage-site of α -secretases. However, on prolonged treatment with ASs, $A\beta$ gets fragmented on many other sites as well, leading to the total destruction of amyloid. The selective nature of ASs was confirmed using a negative control experiment with 28 membered mutant diphtheria toxin (DTP28) protein segment replacing ⁸Val and ²³Lys by Pro.

The kinetics of amyloid accumulation in the form of fibrils was monitored *in vitro* by many biophysical tools; e.g., ThT-assay, TEM, Congo red birefringence, AFM, and DLS study. The results indeed indicated that the $A\beta$ -fragment and the full-length $A\beta$ both did not aggregate in the presence of ASs while they formed fibril in the absence of ASs. As a representative example, the results from a time-dependent TEM and Congo red birefringence study are presented in Figure 2.

Furthermore, similar experiments indicated that one of the ASs, AS4, could indeed destroy pre-formed fibrils and amyloids of $A\beta_{12-21}$ fragment and $A\beta_{1-40}$ peptide. A vesicle leakage study confirmed that AS4 disrupted the preformed amyloid of $A\beta$ into smaller oligomers or monomers that can not form pore in large unilamellar vesicles, thus probably AS4 generated non-toxic metabolites after disruption of $A\beta$ amyloid.

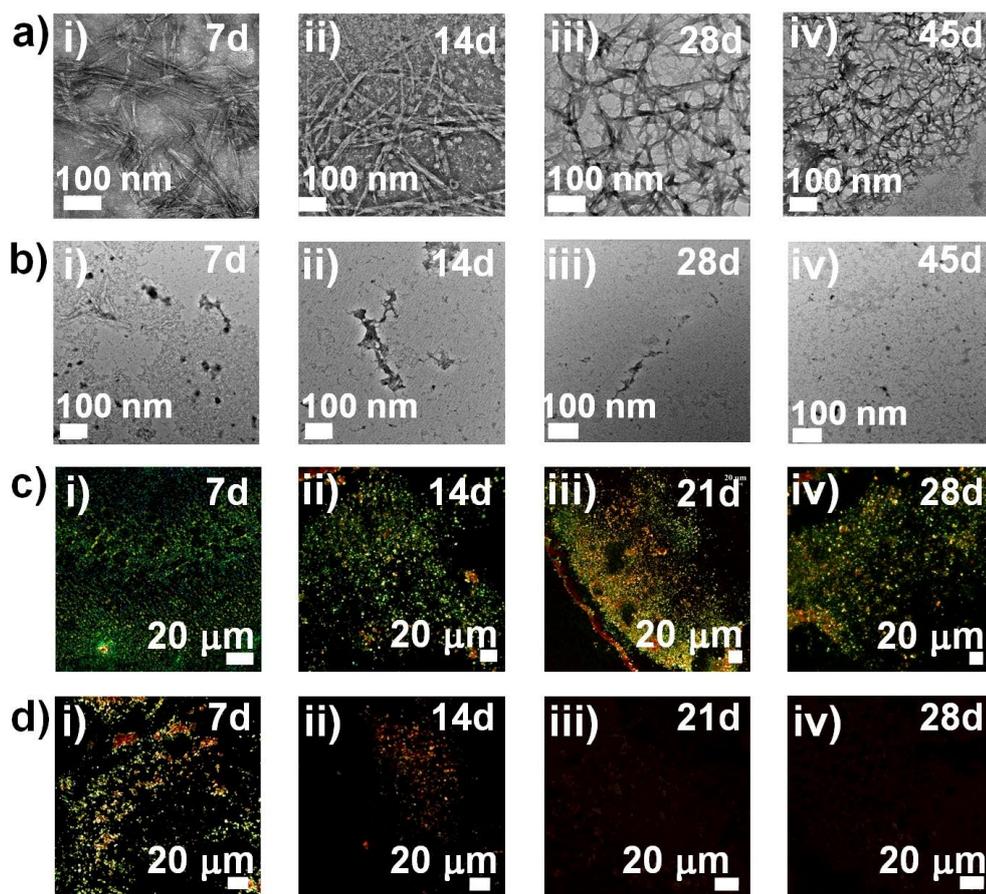


Figure 2

Discussion: The above results indicated that we indeed could develop some peptide-based constructs (ASs) that selectively recognized APP or A β and cleaved them site-selectively at or near the α -secretases-cleavage site. *In-situ* trans-amidation and imide bond formation mechanism were found to play important roles towards the self-assembly driven proteolysis. ASs are supposed to mimic the APP processing activity of α -secretase specifically, leaving all other substrates of α -secretases unaffected; therefore, the medicines developed following this concept may have reduced side effects. Usually, α -secretases cleave soluble folded APP, but cannot metabolize aggregated A β due to its solvent inaccessibility. However, the ASs are designed to act by self-assembly driven proteolysis, therefore, can truncate and dissolve aggregated A β as well. It was indeed noted that the prolonged treatment of A β aggregates with AS4 resulted in further chemical degradation of preformed amyloids.

Conclusion: A robust mechanism of the total destruction of Amyloid has been established. The mentioned peptidic constructs may lead to a robust strategy for the intervention of amyloidosis that in turn may help in drug design against not only AD but also other amyloidoses. However, the catalytic feature of the proteolytic enzymes could not have adhered in the *de novo* designed “artificial α -secretases”. Therefore, we are further developing strategies for targeted degradation of amyloid-beta aggregates having proteolytic enzyme-like activity by peptide engineering.

References:

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Repurposing pyrazolones as proteasome activators with neuroprotective activity.

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The threat of amyloid diseases, including Alzheimer's (AD), Parkinson's (PD), and type 2 diabetes (T2DM), concerns the developed world and exacts a poorly documented fee from underdeveloped countries. So far, the prevailing hypothesis has been that amyloid proteins aggregate to form toxic oligomers eventually leading to cell death. But, with so little advancement made so far toward finding a cure, it is time to rethink the path to progress. In particular, it seems clear that the problem must be concurrently approached from a comprehensive cell biochemical perspective, focusing on all the elements of the proteome maintenance machinery including the unfolded protein response, the ubiquitin-proteasome system (UPS) and autophagy. Many researchers have therefore screened several molecules targeting the UPS (e.g. proteasome activators, inhibitors of deubiquitinating enzymes DUBs or ubistatins), proteases or membrane-porating amyloids. Unfortunately, very few molecules have shown to have the potential to be pipelined to clinical trials. Therefore, despite the considerable commercial value of effective disease-modifying therapy, this area is considered as a high-risk one within the pharmaceutical industry.

Aims

Inspired by recent reports showing that some disused painkillers known as pyrazolones may rescue proteasome activity in mice and attracted by the possibility to repurpose "old" medicines and thus de-risk the drug development process, we scrutinized a small library of drugs by assaying their ability to augment proteasome activity and protect neurons from amyloid toxicity.

Methods

Proteasome activity was monitored by the release of fluorescent probes after cleavage of a fluorogenic peptide substrate and by ESI-MS spectrometry. Native gel proteasome assemblies were separated and visualized by probing the gel with fluorescent probes. Docking calculations described the lowest-energy binding poses of proteasome activators, then validated by STD-NMR. The neuroprotective effects of aminopyrine were assayed in vitro (SH-SY5Y cells) and in vivo (3×Tg-AD mice).

Results

Enzyme assays showed that three out of the 12 molecules tested (i.e. aminopyrine, 4-aminoantipyrine and nifenazone) are proteasome activators with aminopyrine being the most active one. Native gel analysis of protein extracts of neuron-derived dopaminergic cells treated with aminopyrine evidenced that this drug activates the 20S proteasome Core Particle (CP) and does not disassemble single (26S) or double (30S) capped proteasome particles to raise free 20S pool. Enzyme assays performed on a permanently "open gate" mutant ($\alpha 3\Delta N$) proteasome showed that aminopyrine activates latent CP through binding the α -ring surfaces and influencing gating dynamics. Docking studies coupled with STD-NMR experiments showed that H-bonds and π - π stacking interactions between active pyrazolones and the enzyme may play a key role in bridging $\alpha 1$ to $\alpha 2$ and, alternatively, $\alpha 5$ to $\alpha 6$ subunits of the outer α -ring.

Conclusions

Aminopyrine exhibits neurotrophic properties and protects SH-SY5Y cells from β -amyloid (A β) toxicity. Preliminary experiments put in evidence a slight dose-dependent rescue of grip strength in 3×Tg-AD mice. Although further studies are needed to fully elucidate the complex mechanisms of action of these molecules, our results point to aminopyrine as a promising compound for the development of proteasome activators to be used in AD therapy.

SPONSORS

