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A novel library of single-domain antibodies capable of modulating Tau aggregation

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Objectives: Alzheimer's disease (AD) is a neurodegenerative disorder characterized by the World Health Organization as a public health priority. The pathology is characterized by the aggregation of amyloid-β (Aβ) and Tau protein into amyloid-β plaques and neurofibrillary tangles, respectively. Neuroinflammation is also implicated in AD and is responsible for the secretion of alarmins, which include the S100B protein. S100B is highly studied in the context of AD, and it's known for its dual function as a detrimental pro-inflammatory mediator and a beneficial anti-aggregation chaperone over Aβ and tau, making it an amenable drug target. Since there is still no cure for AD and is highly attractive to target chaperones with an already reported anti-aggregation activity, a library of single-domain antibodies (or nanobodies) targeting S100B was developed to potentiate S100B chaperone activity and modulate tau aggregation.

Methods: Here, we employed ThT-monitored kinetics of heparin-induced K18 (Tau244-372) aggregation to study the effect of nanobodies alone and in combination with S100B on K18 aggregation. Moreover, we performed mechanistic analysis to determine which step of the aggregation reaction are targeted by the nanobodies.

Results: Several nanobodies potentiated S100B inhibitory effect over K18, possibly by harnessing S100B in a more competent conformation to bind K18. Surprisingly, control experiments revealed that some nanobodies alone significantly inhibit K18 aggregation even at sub-stoichiometric ratios. Further, mechanistic analysis demonstrates that different nanobodies target multiple steps of K18 fibrillation.

Discussion/Conclusion: Anti-S100B nanobodies anti-aggregation activity over K18 aggregation is discussed in the context of possible heterotypic interactions between the nanobody CDR3 region and Tau/K18. These findings uncover the therapeutic potential of anti-S100B nanobodies, which can be used as modulators of K18 aggregation or as activators of S100B chaperone activity.

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Multimers of a brain-expressed alarmin suppress Amyloid-β conformers implicated in Alzheimer´s Disease

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Introduction: Alzheimer's disease (AD) involves extracellular aggregation of Aβ42 into toxic oligomers and fibrils, whose emergence is regulated by a limited set of molecular chaperones. These include the S100B alarmin, a homodimeric EF-hand protein with intra and extracellular functions which acts as a Ca^{2+}-switched Aβ42 anti-aggregation chaperone. However, S100B occurs in the brain also as a homotetramer, with uncharacterized neuroprotective roles.

Objectives: Here, we intended to compare the chaperone activities of both dimeric and tetrameric S100B against Aβ42 aggregation, while exploring the impact of such multimers on the formation of Aβ42 oligomers (AβO).

Methods: S100B anti-aggregation activity was evaluated by thioflavin-T (ThT) Aβ42 aggregation assays. Aβ42 conformers targeted by S100B were accessed by computational and structural-biophysical spectroscopies. Aβ42 oligomer distributions were determined through mechanistic analysis of fibril formation and via early detection of Aβ42 species using the X-34 fluorophore.

Results: Aβ42 aggregation kinetics revealed that, unlike the dimer, tetrameric S100B delays Aβ42 aggregation and reduces the amounts of fibrils formed at sub/equimolar ratios, an effect that persists even in the absence of Ca^{2+} binding. Structural analysis revealed that this enhanced catalytic efficiency results from a secondary Ca^{2+}-independent binding site formed upon tetramerization of S100B, with which monomeric and fibrillar Aβ42 interact. Kinetic and mechanistic analysis revealed that dimeric and tetrameric S100B preferentially inhibit Aβ42 fibril surface-catalyzed nucleation, decreasing the reactive influx towards oligomers down to <10%. Such results comply with an independent screening of AβO using a combination of the thioflavin-T and X-34 fluorophores.

Discussion/Conclusion: Our study sheds new insights on the functional landscape of S100B chaperone multimers, suggesting their critical role in the regulation of proteopathic Aβ42 aggregation and oligomerization in AD. In particular, we found that S100B tetramerization originates a novel Ca^{2+}-independent Aβ42 binding-cleft, which affords sub-molar potency for S100B anti-aggregation and anti-oligomerization activities. Ongoing research on brain-related S100 proteins reveals a novel network of chaperones able to mitigate protein aggregation and toxicity, likely relevant during AD prodromal stages.

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Chaperone-regulated modulation of Tau liquid-liquid phase separation relevant in tauopathies

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Introduction: The phenomenon of liquid-liquid phase separation (LLPS) involving tau is increasingly acknowledged as a contributory process in the onset of tau aggregation and the generation of pathogenic conformers within Alzheimer's disease (AD) and other tauopathies. Neuroinflammation accompanies tau pathology, with late-stage astrocyte-released alarmin exacerbating the condition, while early inflammatory responses encompass protective functions. This applies to the Ca^{2+}-binding protein S100B, which we recently implicated as a proteostasis-regulator that inhibits amyloid-beta and tau aggregation/seeding. These findings suggest a broad holdase-type chaperone function for S100B in counteracting the malformation of protein structures.

Objectives: Our study aims to characterize the regulatory functions of S100B over protein condensation phenomena through its Ca^{2+}-dependent activity as a modulator of tau LLPS induced by crowding agents (PEG) and metal ions (Zn^{2+}). Additionally, we assessed the incorporation of S100B in droplets to exert its regulation functions. Also, we evaluated if the co-localization of S100B with tau within droplets would maintain the liquid-dynamic properties of these membraneless-structures.

Methods: PEG-induced tau LLPS was followed by spectroscopy measurements of light absorbance (400nm) and by fluorescence microscopy using fluorescently labelled tau. Co-localization of S100B within tau droplets was achieved by Förster resonance energy transfer by fluorescence lifetime imaging (FLIM-FRET) using fluorescence-labelled proteins. Evaluation of droplet fluidic characteristics encompassed fluorescence recovery after photobleaching (FRAP) and observation of fusion events.

Results: Phase diagrams indicate significant suppression of tau droplet formation by Ca^{2+}-S100B, without compromising droplet dynamics and liquid properties. Introduction of Ca^{2+} to PEG-induced LLPS with apo-S100B promptly reduces tau droplet levels, highlighting the dynamic, calcium-triggered nature of Ca^{2+}-S100B's action. Likewise, S100B effectively halts PEG-free Zn^{2+}-induced tau LLPS due to its combined Zn^{2+}-buffering and tau-interaction capabilities.

Discussion/Conclusion: Our results establish S100B as a calcium-dependent suppressor of tau LLPS. Collectively, these findings suggests that S100B, functioning as a chaperone, regulates the formation of various pathological conformers and phase-separated systems, strengthening its pivotal role as a proteostasis regulator in early neurodegeneration.

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Mechanisms of protein dysfunction in mitochondrial leukodystrophies: studies on glutamyl-tRNA synthetase (EARS2)

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Introduction: Mitochondrial diseases are the most common group of inherited metabolic disorders characterized by dysfunctional mitochondria due to defects in oxidative phosphorylation. Clinically these diseases are very heterogeneous occurring in all age groups and presenting a wide variety of clinical symptoms, thus delaying diagnosis, disease management and application of targeted therapies.

Mitochondrial aminoacyl-tRNA synthetases (mt-aaRS) are essential proteins for the translational process in the mitochondria, whose defective function leads to mitochondrial disorders, which are often neurological related, such as leukodystrophies. Although in recent years numerous disease variants have been identified in mt-aaRS, the molecular basis of the associated pathologies is not yet clear, which is a major gap in the field of mitochondrial disorders. Aiming at contributing to the clarification of the molecular mechanism of leukodystrophies associated to mt-aaRS defects we designed a project focusing on glutamyl-tRNA synthetase 2 (EARS2), a mt-aaRS that catalyzes the addition of glutamate to its mitochondrial tRNA(Glu).

Objectives: The objective of my master project is to study EARS2 disease variants (EARS2-p.Glu96Lys, EARS2-p.Gly110Ser, and EARS2-p.Arg489Gln) to uncover possible genotype-phenotype correlations.

Methods: Recombinant expression in *E. coli* of the human EARS2-wt and disease variants, and subsequently purification of the target proteins using a combination of different chromatographic techniques. The pure proteins will be characterized regarding structure, stability, and function by biochemical and biophysical methodologies, such as circular dichroism, fluorescence spectroscopy and differential scanning fluorimetry.

Results: EARS2 wild-type protein was expressed in *E. coli* cells and purified with the purity yield higher than 90%. Analysis of secondary and tertiary structure of EARS2 wild-type revealed that the purified protein presents a folded conformation, with the expected α-helical fold. Further, the thermal unfolding process of this protein is cooperative, and the results obtained by different methods indicate that EARS2 loses its tertiary structure, and then secondary structure is altered. Currently, I am dedicated to the studies on disease variants.

Discussion/Conclusion: We expect our results to provide new information regarding EARS2 molecular defects and could, in the future, open new avenues to improve disease prognosis and therapies.

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A New Sticker on the Block: Novel Bioadhesives Inspired by Sea Urchin Adhesive Proteins

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Introduction: Currently, there is a great need in biomedicine and biotechnology for biological adhesives that are non-cytotoxic and efficient in aqueous environments (i.e., surgical adhesives or cellular adhesion promotors for *in vitro* cultures). It is already known that marine invertebrates can attach to several substrates in the presence of seawater (high dielectric and ionic strength similar to physiological fluids) through the production of protein-based adhesive secretions. Some have already inspired the development of new biomimetic adhesives (i.e. Cell-Tak™, a formulation containing proteins from marine mussel). However, successful examples are still scarce and there is still a need for new adhesive systems, with novel capabilities. Considering this, our group has been studying sea urchins' adhesion, focusing on *Paracentrotus lividus.* Nectin was identified as an important adhesive protein in this species, present in both adhesive organs and secretions. It has six galactose-binding discoidin-like (DS) domains, which are thought to be important for its adhesive function.

Objectives: Our main goal is to develop a new bioadhesive inspired by sea urchins' Nectin. To do so, we will study the full-length Nectin protein and design several combinations of its DS domains, aiming at producing a stable adhesive protein for large scale production.

Methods: Therefore, we will use recombinant protein expression in *E. coli* cells. The best targets will be purified using a combination of chromatographic methodologies and protein quality will be evaluated using biochemical methods. For the top targets, a structural and conformational characterization will be performed using techniques like circular dichroism, fluorescence spectroscopy and differential scanning fluorometry. Adhesiveness will be assessed through surface coating analysis and atomic force microscopy.

Results: Production of the full-length protein is still ongoing. Regarding the constructs, four of the six designed were successfully expressed. One of them was purified with a 90-95% purity and characterized. This construct demonstrated to be fairly thermal stable and presented a folded conformation with a typical α/β structure.

Discussion/Conclusions: This project can contribute to the current knowledge on *P. lividus* adhesion mechanisms and open new avenues for the development of sea urchin inspired bioadhesives for biomedical/biotechnological applications.

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