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Abstract Booklet

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Tau liquid-liquid phase separation is regulated by the calcium-binding S100B chaperone

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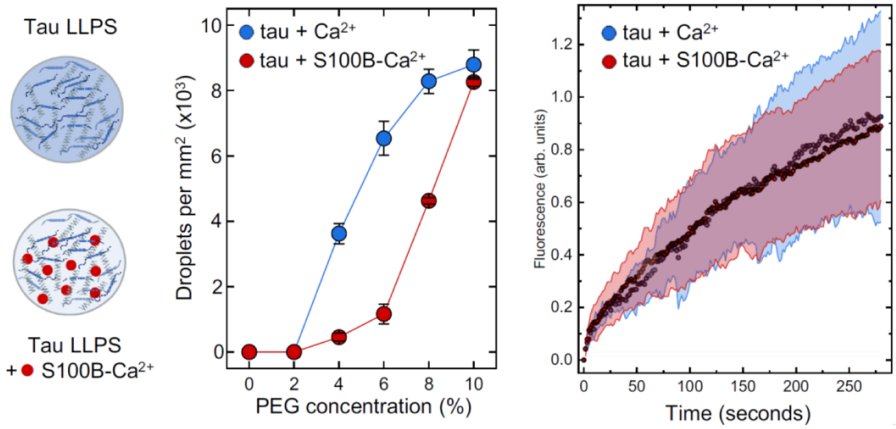
The phenomenon of liquid-liquid phase separation (LLPS) involving tau is increasingly acknowledged as a contributory process in the onset of tau aggregation and the generation of pathogenic conformers within Alzheimer's disease (AD). Neuroinflammation accompanies tau pathology, with late-stage astrocyte-released 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 (Cristovão et al 2018 Sci Adv) and tau aggregation/seeding (Moreira et al 2021 Nat Commun). These findings suggest a broad holdase-type chaperone function for S100B in counteracting the malformation of protein structures. Our study aims to elucidate S100B's role in tau LLPS. PEG-induced tau LLPS was followed by spectroscopy measurements of light absorbance (400nm) and tau fluorescently labelled. Co-localization of S100B within tau droplets was achieved using fluorescence-labelled proteins and FLIM-FRET. Evaluation of droplet fluidic characteristics encompassed fluorescence recovery after photobleaching (FRAP) and observation of fusion events. Phase diagrams indicate significant suppression of tau droplet formation by Ca^{2+}-S100B, preserving droplet 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. 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. Acknowledgments: EU for funding Twinning Grant EU-TWIN2PIPSA/GA101079147, FCT/MCTES (Portugal) for funding UIDB/04046/2020 and UID/MULTI/04046/2020 (BioISI) and PhD grant 2020.06443.BD (GGM) and Agilebio for funding LabCollector Scientific Award 2021 (CMG).



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Deciphering Protein Misfolding in Inborn Errors of Metabolism: Insights from Structural Biology

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Inborn Errors of Metabolism (IEM) belong to the group of rare diseases and are associated to defects in enzymes involved in different metabolic pathways. Although individually rare, they are collectively quite numerous and affect millions of people worldwide. The vast majority arise from single gene mutations that disrupt cellular metabolism, frequently resulting from faulty protein folding. While the genomic era has facilitated the identification of more cases, establishing a clear correlation between genotype and phenotype remains challenging.

To contribute to a better molecular understanding of IEM in the last years we have been investigating disease-related variants combining biochemical, biophysical and structural methods to establish the effects of point mutations on protein folding, stability and function. Here, we highlight our recent investigations in Multiple Acyl-CoA Dehydrogenase Deficiency (MADD) and Glutaric Aciduria-type I (GA-I).

MADD results from deficiencies in the alpha or beta subunit of electron transfer flavoprotein (ETF), or in the ETF:ubiquinone oxidoreductase (ETF:QO), and is associated to impaired mitochondria beta oxidation. Our biochemical and structural analyses of ETF:QO variants offer a molecular basis for understanding the reduced enzyme activity observed in patients [1, 2].

GA-I is a neurometabolic disorder caused by deficiency of glutaryl-CoA dehydrogenase (GCDH). By conducting studies on two disease variants (GCDH-p.Arg227Pro and -p.Val400Met), we elucidated genotype-phenotype correlations, revealing that both variants maintain the overall protein fold while exhibiting distinct conformational stabilities [3]. Furthermore, compromised enzymatic activity on both proteins depend on different features [3]. Additionally, we have provided a molecular explanation for the potential efficacy of riboflavin supplementation in GA-I patients [4].

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Heterotypic Interactions Drive Anti-Aggregation Activity of Nanobodies Against S100B on Tau Aggregation

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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. Here, we employed ThT-monitored kinetics of heparin-induced K18 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. Several nanobodies potentiated S100B inhibitory effect over K18 (Tau244-372), 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. This striking observation is discussed in the context of possible heterotypic interactions between the nanobody CDR3 region and Tau/K18. Further, mechanistic analysis demonstrates that different nanobodies target multiple steps of K18 fibrillation. 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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Exploring Early Extracellular Disease Mechanisms: Cross-Interactions Between Tau and Amyloid β in Alzheimer's Disease

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Since the underlying mechanisms of early Alzheimer's Disease are not well understood, we aimed to explore plausible extracellular cross-interactions between Tau and Amyloid beta (Aβ) that could condition disease development, as well as the effect of the early-stage extracellular chaperone S100B in this context. The effect of Aβ on Tau was assessed following the aggregation of a fixed concentration of the Tau AD core (TADC, Tau306-378) by monitoring X-34 fluorescence intensity in the presence of Aβ42 monomers under cofactor-free conditions. To study the effect of Tau over Aβ, the in vitro aggregation of a fixed concentration of Aβ42 monomers in the presence of increasing proportions of TADC and Tau-K18 (Tau244-372) was monitored by ThT fluorescence. Finally, the influence of S100B on TADC aggregation, with and without heparin, was similarly evaluated with the fragment alone and in the presence of Aβ42 monomers. The toxicity of end-point species was assessed by liposome leakage assays. TADC aggregation was accelerated in the presence of Aβ42 in a concentration-dependent manner, and the end-point species present were fully toxic for liposomes. On the contrary, Aβ42 aggregation was inhibited by both TADC and K18. S100B had a dual-behaviour on TADC aggregation, accelerating it at substoichiometric proportions and fully inhibiting it at equimolar proportions, though this inhibition was not complete when heparin was present. Finally, under mixed conditions with Aβ42, the inhibitory effect of S100B over TADC aggregation was lost. In conclusion, Tau and Aβ have opposite effects on each other's in vitro aggregation. S100B, while able to inhibit TADC aggregation and toxicity, showed a concentration-dependent dual-behaviour, accelerating its aggregation at lower proportions. These results highlight the complex interplay of events in early-disease scenarios in Alzheimer's Disease.

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Unravelling Molecular Mechanisms of Leukodystrophies Associated to gGutamyl-tRNA Synthetases (EARS2) Disease Variants

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Mitochondrial diseases (MD) are the most common group of inherited metabolic disorders characterized by dysfunctional mitochondria due to defects in oxidative phosphorylation. In recent years the interest around mitochondrial aminoacyl-tRNA synthetases (mt-aaRS), a family of enzymes that ensure proper translation by adding the appropriate amino acid into the correct tRNA molecule, is emerging due to the increasing number of MD patients with mutations on these enzymes, including in Portugal. Although the knowledge around these disorders has been increasing, there are still open questions including the fact that the characterization of human mt-aaRS at the protein structural, conformational and functional levels is scarce.

Aiming at contributing to the clarification of the molecular mechanism of leukodystrophies associated to mt-aaRS defects here we present our recent work on glutamyl-tRNA synthetase (EARS2) wild-type and three disease variants. We have used protein biochemical and biophysical methodologies to make for the first time, to our knowledge, a structural characterization of human EARS2 protein.
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 structure. Further, the thermal unfolding process of this protein is cooperative, with an apparent Tm of ~45°C. Currently, we are dedicated to the studies on the three disease variants.

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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Biomimetic Adhesives: New Adhesive Proteins Inspired in Sea Urchin Nectin Structural Domains

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Currently, there is a great need in biomedicine and biotechnology for biological adhesives that are non-cytotoxic and efficient in wet/humid environments (i.e., surgical adhesives or cellular adhesion promotors for in vitro cultures). It is already known that marine invertebrates are able to attach to several substrates in the presence of sea water (high dielectric and ionic strength similar to physiological fluids) through the production of adhesive secretions. Some of which 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.

Sea urchins bioadhesives are an ideal framework to develop new bioinspired adhesives due to their adhesion strengths up to 0.5 MPa, which are higher than commercially available biological adhesives (e.g., fibrin glue 0.2 MPa). Our group has been studying sea urchins' adhesion, focusing on Paracentrotus lividus. For this species, Nectin was identified as an important adhesive protein 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. Aiming to develop a new synthetic bioadhesive inspired in sea urchins' Nectin, we are currently studying the full-length Nectin protein and several combinations of its DS domains (constructs), to obtain an adhesive, stable protein for large scale production. To do so, several E. coli strains, and growth conditions were tested. The best targets were purified using a combination of chromatographic methodologies, and protein quality was evaluated using biochemical methods. For the top targets, a structural and conformational characterization was obtained through circular dichroism, fluorescence spectroscopy and differential scanning fluorometry. Adhesiveness will be assessed through surface coating analysis and atomic force microscopy.

By now, we were able to express the full-length protein and 4 out of 6 constructs. Furthermore, at least one construct was purified with a 90-95% purity and structurally characterized. Circular dichroism spectra indicate that the purified construct presents a folded conformation. And monitoring loss of secondary structure, or tryptophan fluorescence emission during thermal stress showed a fairly thermal stable protein. Providing a deeper characterization of the first identified sea urchin adhesive protein, this project contributes to the current knowledge on P. lividus adhesion mechanisms and opens new avenues for the development of sea urchin inspired bioadhesives for biomedical/biotechnological applications.

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Chaperone multimers suppress the generation of Aβ42 neurotoxic oligomers implicated in Alzheimer´s Disease

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Alzheimer's disease (AD) involves extracellular aggregation of Aβ42 into toxic oligomers and fibrils, whose emergence is regulated by molecular chaperones. These include S100B alarmin, a homodimeric EF-hand protein with intra and extracellular functions which acts as a Ca2+- switched Aβ42 anti-aggregation chaperone. However, S100B occurs also as a homotetramer, with uncharacterized neuroprotective roles. Here, we compared the chaperone activities of both S100B multimers and explored their impact on the formation of Aβ42 oligomers (AβO). 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. 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 (Figueira et al JMB 2022). 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 (Figueira et al Front. Neurosci. 2023). Our study sheds new insights on the functional landscape of S100B chaperone multimers, suggesting its critical role in the regulation of proteopathic Aβ42 aggregation and oligomerization in AD.

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Succinylation's impact on MCAD enzyme - from structure to function

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Metabolic regulation encompasses a complex interplay of genomic, proteomic, and metabolomic adjustments within cells. A particular group of non-enzymatic post-translational modifications (PTMs) known as acylations, such as succinylation and glutarylation, have emerged as important regulators in mitochondrial enzymes [1].

The extent of acylations is closely associated with the accumulation of intermediate metabolites such as succinyl-CoA and glutaryl- CoA that occur under certain conditions such as fasting, caloric restriction or in several metabolic disorders, creating a unique scenario for anomalous protein acylation [2]. It has been recently shown that the enzyme glutaryl-CoA dehydrogenase (GCDH), which participates in amino acid catabolism, is prone to high levels of glutarylation due to an increase in glutaryl-CoA production stimulated by lysine catabolism, and this modification diminish enzyme activity [3]. Moreover, it was demonstrated that this acylation was regulated by sirtuin5 (Sirt5). Exploring this new concept which suggests that enzymes involved in pathways that handle these metabolites are more prone to protein acylation, in this work we combine biochemical and biophysical techniques to address the impact of succinylation on the structure and function of the Medium Chain Acyl-CoA Dehydrogenase (MCAD) enzyme.

Our results show that purified MCAD is easily succinylated and glutarylated. Through spectroscopic techniques such as circular dichroism or fluorescence we were able to demonstrate that succinylation has no major implication on MCAD's structure. Further using thermal denaturation assays, we also access protein thermal stability, and observe no major changes due to succinylation. Nonetheless, this modification induces an increase of enzymatic activity. In addition, we show that SIRT5 incubation reverts succinylation and brings function to similar levels to unmodified MCAD, in agreement with studies in other proteins.

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