Laidlaw Research Reflective Report

Investigating the Role of Alpha synuclein Charge in Synaptic Vesicle Fusion and Neurotransmitter Release Using Mass Spectrometry

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BACKGROUND OF THE SUBJECT

Over the past two decades, interest in α-synuclein protein research has grown significantly and continue to increase due to its central role in the pathogenesis of synucleinopathies- a group of neurodegenerative diseases caused by the aberrant aggregation of α-synuclein, most notably Parkinson's disease (PD). α-synuclein is an intrinsically disordered protein that lacks a well-defined globular structure which is expressed abundantly in the central nervous system and located mainly at the presynaptic terminals. It is the main protein component of Lewy bodies and Lewy neurites-protein aggregates that are also abundant in lipids-and is a main neuropathological hallmark of Parkinson's disease and dementia (Makasewicz et al., 2024). Although the exact role of this protein in disease pathogenesis processes is not well established, it is associated with dopamine releases and vesicular trafficking, as well as play role in membrane phospholipids and proteins interaction to regulate synaptic plasticity and neurotransmission (Sarchione et al., 2021).

Structurally, α-synuclein has three distinct regions within the primary amino acid sequence such as N terminal region (1-60 residue), NAC domain (61-95 residue), and C-terminal (Moons et al., 2020). The first region constitutes a high number of positively charged residues, and it takes on diverse extended and more compact conformation. The NAC region is characterized by a high number of hydrophobic residues and constitutes the core of the stacked β-sheet structure, which is formed through intermolecular interactions between NAC regions of adjacent α-synuclein molecules. The C terminal retains disordered conformation and is rich in negatively charged residues. This part functions as a binding site for various cations and is susceptible to truncation and post translational modifications (Emamzadeh, 2016).

The propensity of α-synuclein aggregation can be influenced by intrinsic factors, such as mutations, gene multiplications, post translational modifications, and extrinsic factors, including membrane(lipid) interaction, presence of other molecules and ions (Lomeli-Lepe et al., 2023). Among the various modulators of α-synuclein aggregation, lipid interaction is particularly important, as it involves frequent interaction during physiological roles such as synaptic vesicle trafficking and neurotransmitter releases. This plays a key role in influencing whether the protein adopts a stabilised structure or misfold into pathogenic conformation. However, the exact mechanism in which lipid interactions induce physiological oligomerisation or pathological aggregation of the protein remain unclear. Furthermore, it has also been shown that pathological aggregates of α-synuclein contain the membrane fragments from different cellular organelles (Shahmoradian et al., 2019), reinforcing the idea that lipid interaction is central to disease mechanisms.

α-synuclein does not exhibit strict lipid specificity, nevertheless; it binds more strongly to anionic lipids due to electrostatic attraction between the negatively charged headgroup of lipid and the positively charged N terminal site of the α-synuclein, and hydrophobic interactions (Makasewicz et al., 2024). However, studies have shown that low lipid-to-protein ratio tends to promote the folding of α-synuclein into amyloid fibril, whereas, at higher lipid-to-protein ratios, the protein structure is stabilised, reducing aggregation propensity (Ramirez et al., 2023).

Although studies hypothesised the important interaction of lipid and α-synuclein (Galvagnion, 2017; Battis et al., 2023) there is little detailed understanding at the molecular level on how α-synuclein monomers conformational behaviours change or how it may influence further α-synuclein aggregation pathway. In the current study we employed native mass spectrometry coupled to ion mobility mass spectrometry (nMS-IM-MS) to understand binding interactions of α-synuclein monomer to phosphatidylinositol 4,5-bisphosphate (PIP_2) and phosphatidylinositol 3,4,5-trisphosphate (PIP_3) membrane lipids. We also investigated α-synuclein's interaction with detergents that mimic the membrane environment and H50A variant to PIP_2 and PIP_3 to identify any shifts in confirmation, binding strength, and oligomerisation states.

Furthermore, this study encompasses Epigallocatechin gallate (EGCG) which known for its key role in modulating α-synuclein aggregation through inhibiting fibril formation, stabilising non-toxic oligomers and remodelling of existing fibril into non-toxic aggregates (Gonçalves et al., 2023; Xu et al., 2016). Prior investigations of EGCG have primarily focused on its interactions with the wild-type protein, leaving the effect on disease relevant mutants poorly understood. Therefore, we explored whether the EGCG binds to the mutant-type protein (H50A) with similar affinity and critically assessed its modulation of oligomeric states and conformational ensembles.

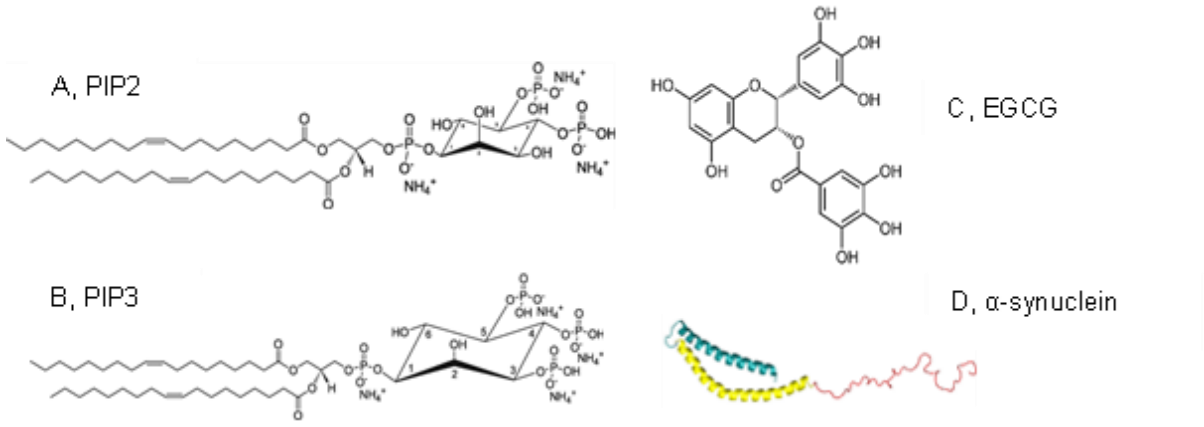

Figure 1, *illustrating the molecular structures of synthetic PIP_2 (1,2-dioleoyl-sn-glycero-3-phospho-(1'-myo-inositol-4',5'-bisphosphate) (ammonium salt) [18:1 $PI(4,5)P_2$), PIP_3 (1,2-dioleoyl-sn-glycero-3-phospho-(1'-myo-inositol-3',4',5'-trisphosphate) (ammonium salt) [18:1 $PI(3,4,5)P_3$), EGCG (Epigallocatechin gallate) and α-synuclein which are employed in this study.*

Native mass spectrometry (native nano electrospray) enables us to detect the binding of α-synuclein with PIPs, detergent and EGCG molecules by showing a distinct shift in the protein mass, while preserving the non-covalent interaction that are often disrupted in conventional intact mass analysis. Additionally, integration of Ion mobility will allow us to detect different conformational ensembles of α-synuclein in the presence of PIP_2, PIP_3, detergent micelles and EGCG. This can be identified by analysing their drift time, which reflects the relative mobility of ions in the gas phase and provides insights into how compact or extended each conformation is.

Why is this study impactful?

This research encompasses a broad range of meaningful impact in the scientific community. Although α-synuclein is among extensively studied intrinsic disordered proteins, its exact mechanism in disease pathogenesis is not clear, posing a great challenge in terms of therapeutic solutions. However, research like this provides a new insight into how α-synuclein specifically interacts with signalling lipids such as PIP_2 and PIP_3 through capturing the binding and structural dynamics of these complexes in a near-native state. This has potential relevance, as disrupting or modulating lipid–protein interactions may represent a novel strategy to slow or prevent α-synuclein aggregation and disease progression.

Furthermore, as the research investigates α-synuclein mutants' interaction with EGCG under distinct biologically relevant conditions, it reveals how genetic variation alters EGCG bindings compared to the wild type and examines whether it can modulate or alter any conformational state. This approach fills the gap in the field as no well-established understanding exists on how the experimental mutant(H50A) α-synuclein interacts with EGCG. These findings will contribute to the deeper understanding of interactions at the molecular level, providing insights into potential intervention strategies in the avenue of drug development and or hold a significance in guiding future research directions.

Methodology

Recombinant α-synuclein (100 μM) and the H50A variant were buffer-exchanged into ammonium acetate using a Micro Bio-Spin P6 spin desalting columns prior to native mass spectrometry. The storage buffer present in the Bio-Spin column was removed by centrifugation (2 min at 1g), followed by 3-5 equilibration spins with 500 μL of 50mM ammonium acetate (pH ~7.0). The protein sample was then applied and recovered by a final centrifugation step at 1g for 4min. Protein concentration was determined by NanoDrop A280 using an ammonium acetate as blank.

A 10 μM stock solution of α-synuclein was prepared for further experiments. A total of 100 mg of both PIP_2 and PIP_3 (purchased from Avanti Polar Lipids, Inc.) was mixed with 100 μL of methanol to facilitate dissolution in aqueous solutions. The solution was exposed to a stream of nitrogen gas to evaporate the methanol, and the resulting lipid films were reconstituted in desired volumes of 50 mM ammonium acetate to create a biologically relevant environment for protein interaction. Both wild-type and mutant-type α-synuclein were mixed with each lipid and EGCG drug molecules at a molar ratio of 1:20 (protein: ligand) and incubated on ice for 30 minutes.

Sodium iodide is also used as a calibrant, as it forms predictable ion clusters across a wide mass range, allowing accurate calibration of the instrument's mass scale. Standard proteins such as myoglobin, cytochrome c and β-lactoglobulin of 20 μM were prepared and sprayed to calibrate ion mobility cell (Synapt G1) prior to spraying the samples.

Results:

Interaction of phosphatidylinositol 4,5-bisphosphate (PIP_2) and phosphatidylinositol 3,4,5-trisphosphate (PIP_3) with *α-Synuclein.*

The native mass spectrometry data revealed key electrostatic interactions and conformational changes of α-Synuclein across multiple charge states (10+ to 7+). Apo α-synuclein displays a clean charge-state ladder with no adducts (Figure 2/ green). Upon adding PIP_2 (black) and PIP_3 (blue), new peaks appear at the expected lipid-dependent mass offsets, consistent with 1-2 PIP_2 and PIP_3 molecules bound per monomer. The observation of equal numbers of PIP_2 and PIP_3 bound states across the charge state (10+ to 7+), suggests that the protein has the same potential binding sites for either of the lipids. This is consistent with previous reports indicating that α-synuclein exhibits no clear lipid preference, with its interactions across various experimental lipid models being well established (Makasewicz et al., 2024). This observed binding of α-synuclein with multiple PIP_2 and PIP_3 is likely driven by the electrostatic interaction between the lysine rich N terminal domain and the highly anionic phosphate group, and Hydrophobic interactions between the NAC region and the acyl chains of PIPs may further stabilize these complexes (Jao et al., 2008; Narayanan and Scarlata, 2001).

Although the same number of lipids bound states are present, the peak intensity for PIP_3 bound states are consistently higher than those for PIP_2, suggesting that PIP3 has higher binding affinity and greater stability of the α-synuclein-PIP_3 complex during ionization and transmission. This result aligns with a prior study reporting that PIP_3 forms more stable and tighter complexes with proteins due to the additional negative charge and enhanced hydrogen bonding capacity (Hammond and Balla, 2015). PIP_3 carries an extra phosphate, which

increases charge density and multivalent contacts with α-syn's N-terminal amphipathic segment, which is also known to drive binding to anionic headgroups.

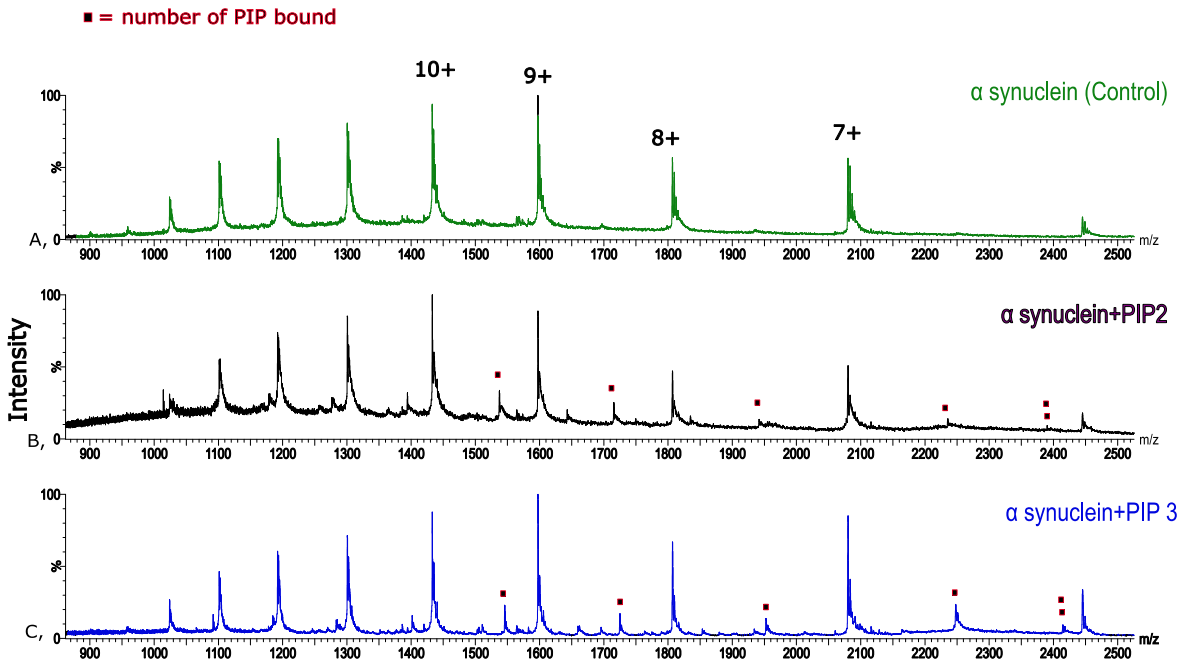

Figure 2.*Native Mass spectra of α-synuclein a) absence of lipids (control) and in the presence of b) PIP_2 and c) PIP_3 at 1:20 protein to lipid molar ratio. An equal number of bound states was observed in each case, although their relative intensities varied.*

Conformational Effects of PIPs binding

Biological interactions are often accompanied by conformational changes in the biomolecule upon binding to small molecules such as lipids. To further elucidate the structural rearrangements induced by PIP binding to α-synuclein, we performed ion mobility mass spectrometry experiments. Ion mobility analysis of α-synuclein revealed pronounced, charge-state dependent structural responses to PIP binding as shown in Figure 3. At 10+, the compact apo population (2.34 ms/ first peak population) shifts toward conformations with larger collision cross sections., stabilising extended state upon binding (PIP_2: 3.97 ms, +69.7%; PIP_3: 4.33 ms, +85%), with PIP_3 shifting the extended further than PIP_2. Whereas the extended apo population (5.95ms/ second peak) shifts only slightly (5.95→6.31 ms). In contrast, at 9+ and 8+, binding produces marked compaction (7.75→5.05 ms, −34.8%; 6.85→4.87/5.77 ms, −28.9%/−15.8% for PIP_2/PIP_3). Unlike other charge states we have also observed two additional PIP molecules bound to α-synuclein at 7+ charge state, where one lipid yields small compaction (PIP_2: 5.59 ms; PIP_3: 5.41 ms) and the two lipids induce expansion (PIP_2×2: 6.13 ms; PIP_3×2: 6.31 ms). This data reflects that PIPs binding remodels the α-synuclein ensemble in a manner that depends on protonation state and lipid stoichiometry-compacting intermediate-charge states while expanding or redistributing the highest-charge and double-bound states.

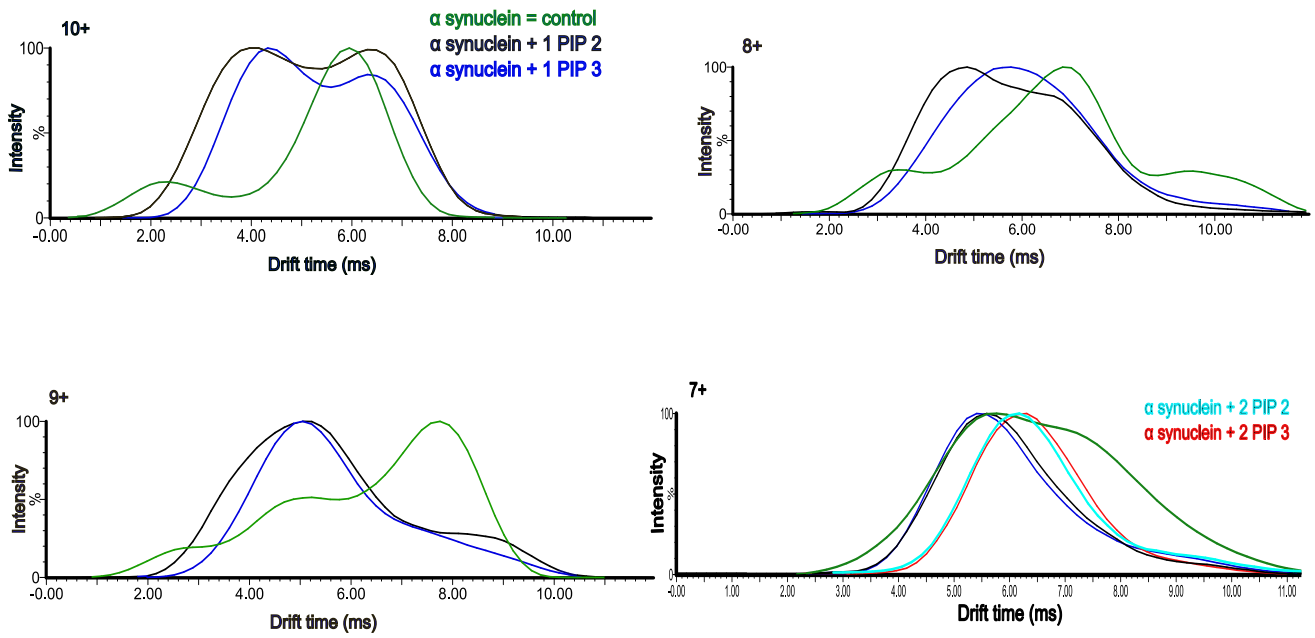

Figure 3.*Drift time plot extracted from native mass spectrometry (N-MS) spectra of Figure1, showing conformational effect of α-synuclein in the presencePIP_2 and PIP_3 interaction across 10+ to 7+ charge state. All plots show drift time distributions corresponding to α-synuclein bound to one PIP_2 and one PIP_3 molecule. For the 7+ charge state, additional drift time populations corresponding to two PIP_2 and two PIP_3 molecules are also detected. Right shifts/shorter drift time indicating compaction and left shift/longer drift time indicating extended state of α-synuclein.*

Interaction of Epigallocatechin gallate (EGCG) with both wild type and mutant(H50A) *α-Synuclein.*

Previous studies have demonstrated that α-synuclein interacts with EGCG (Bieschke et al., 2010; Xu et al., 2016). To investigate the competitive interplay between EGCG and PIP binding, including the effects of α-synuclein mutants, we conducted preliminary experiments with EGCG under optimised conditions. Our study using native mass spectrometry, revealed distinct differences in EGCG binding propensity between wild-type and α-synuclein mutant (H50A) as shown in Figure 4. In the wild-type, EGCG bound peaks are detected, but they are relatively low in intensity (Table1), with the apo species remaining predominant. In contrast, the mutant showed markedly increased EGCG-bound peak intensity and, notably, multiple bound states (up to 2–3 EGCG molecules per monomer) were more prevalent in the mutant than the wild-type, indicating stronger ligand association and a shift in the equilibrium toward the bound form. This suggests that mutation introduces or exposes additional high-affinity binding sites or increases the affinity of EGCG binding regions. This result aligns with prior studies showing that disease-related mutation can destabilise the native conformation (Boyer et al., 2019; Porcari et al., 2015), resulting in more flexible and exposed regions for ligand

binding surface, increasing the binding propensity of the protein by small molecules like EGCG.

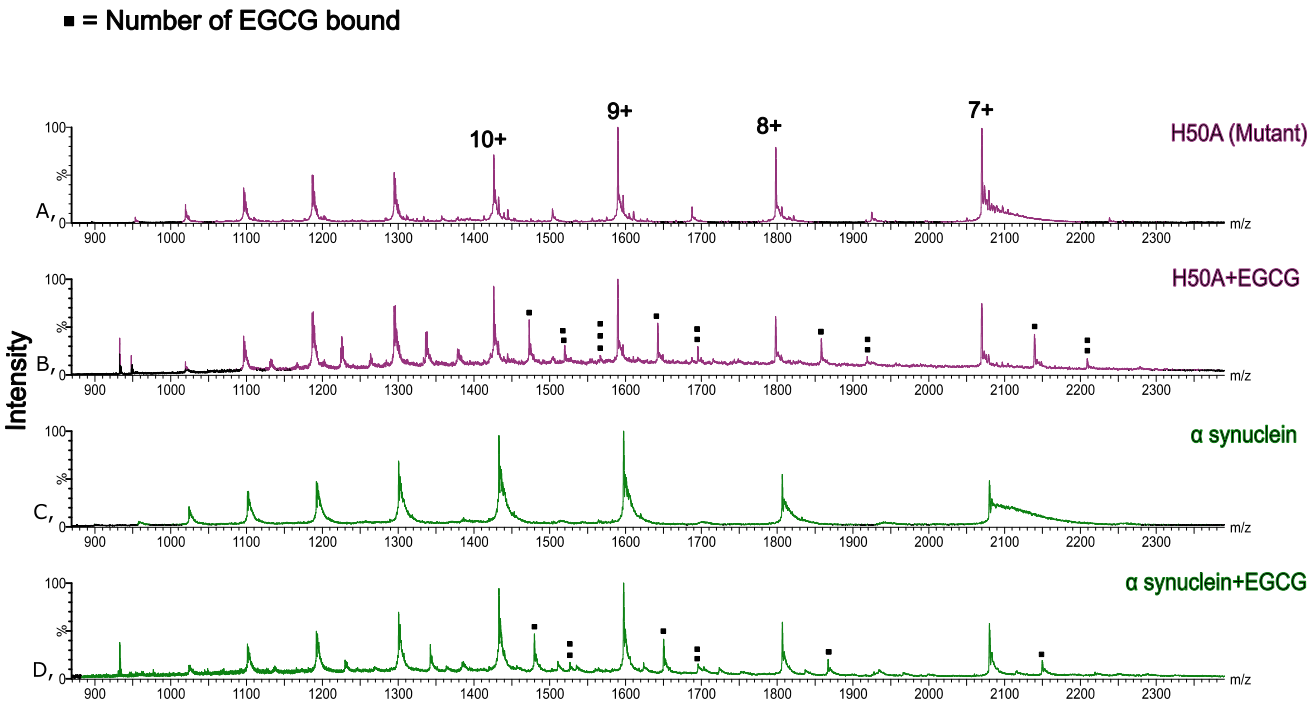

Figure 4, *Mass spectra of 10+ to 7+ charge states of both H50A and wild type α-synuclein, recorded with and without EGCG and were analysed to assess the difference in binding stoichiometry. Varied number of bound states and peak intensities were observed between H50A+EGCG and WT α-synuclein+ EGCG.*

Table 1: *Comparison of the intensity ratio of EGCG bound states between H50A mutant and wild-type α-synuclein at 10+ and 9+ charge states. Ratios were obtained from dividing the intensity of each bound states by the intensity of the apo peak within the same charge state*

Charge state	Number of EGCG bound	H50A intensity ratio	WT intensity ratio	Observation
10+	+1 EGCG bound	**0.63**	0.47	H50A > WT
	+2 EGCG bound	**0.37**	0.21	H50A > WT (~2-fold higher)
9+	+1 EGCG bound	**0.53**	0.40	H50A >WT
	+2 EGCG bound	**0.30**	0.15	H50A > WT (2-fold higher)

Conformational Effects of EGCG binding

In the absence of EGCG, IM-MS analysis (Figure 5) of H50A α-synuclein consistently showed shorter drift time or compact conformation compared with wild type, suggesting that the mutation induced compact conformational ensemble. This is likely due to the loss of histidine-mediated electrostatic interaction or hydrogen bonding at position 50, shifting the balance

toward a compact conformation driven by hydrophobic interaction, which in turn may expose extra binding sites that enhance binding of EGCG as previously described.

IM-MS analysis (Figure 5) of conformational changes in α-synuclein structure upon EGCG binding shows that EGCG alters α-synuclein's conformational ensemble in a stoichiometry- and charge-dependent manner. Wild-type α-synuclein displays modest changes: at 10+ no conformational effect was detected upon EGCG binding (5.77 ms), while at 9+ EGCG introduces a compact subpopulation (5.05 ms) alongside an apo-like species (7.75 ms). Whereas the H50A mutant shows pronounced heterogeneity and formation of very compact species at 10+ charge state with 2 and 3 EGCG molecules bound features a distinct low-drift conformer (3.61 ms), and at 9+ and 8+, multiple conformers appear at both lower and higher drift times relative to apo. These results indicate EGCG stabilizes compact α-synuclein conformers much more readily in the H50A interaction than in wild type.

The appearance of EGCG-stabilized compact conformers, especially the very low-drift species observed for H50A is consistent with prior reports that EGCG binds preferentially to unfolded or aggregation-prone protein conformations where EGCG remodels α-synuclein away from fibril-forming conformers into compact, non-fibrillar oligomers or folded monomers, potentially reducing aggregation risk (Bieschke et al., 2010; Ehrnhoefer et al., 2008). This strong binding and conformational compaction effect of EGCG implies that certain mutants may be more responsive to small-molecule inhibitors that reduce protein aggregation than wild-type, indicating the implication of designing therapeutic strategies depending on the specific structural changes introduced by different mutations.

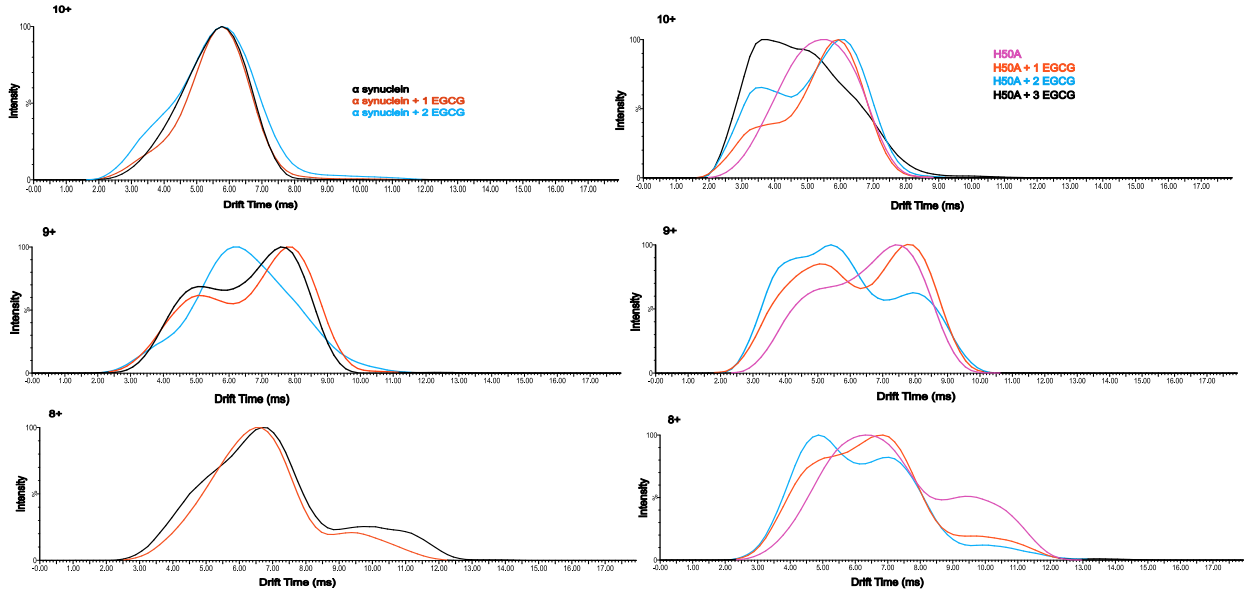

Figure 5, *Drift time plots extracted from Figure 3, showing the conformational states acquired by wild-type α-synuclein (right) and the H50A mutant (left) upon binding to EGCG across the 10+ to 8+ charge states. Each plot illustrates the conformational changes associated with the number of EGCG molecules bound.*

Ongoing and Future Work

In addition to the data presented here, we have also examined the binding of α-synuclein with membrane-mimicking detergent and further investigated the interactions of the H50A α-synuclein mutant with PIP_2 and PIP_3 to identify potential shifts in conformation, binding strength, and oligomerisation states. These analyses are still in progress, and therefore detailed results are not included in this report. The data reported here form part of a broader study that will undergo further analysis and be expanded upon in future publications. Subsequent work will aim to integrate these ongoing investigations to provide a more comprehensive understanding of α-synuclein conformational dynamics and membrane interactions.

Reflection

Activities undertaken

During my summer internship, I engaged in a range of activities that enhanced my technical, social, and academic development. Before starting my project, I conducted extensive background research to deepen my understanding of the role of α-synuclein in normal physiological processes and its pathological involvement in neurodegenerative diseases, particularly Parkinson's disease. Since my research involved the use of a native mass spectrometry-a technique with which I had no prior experience-I dedicated considerable time to learning about the instrument. I watched instructional videos and reviewed previous research data generated using the machine to familiarise myself with its operational techniques and applications. Additionally, I proactively sought guidance from my supervisors, requesting videos and other materials to strengthen my knowledge and ensure I could operate the instrument effectively. These activities significantly improved my confidence and enabled me to learn and understand the research process more efficiently. In turn, this contributed to the overall productivity of the research team by allowing experiments to be executed on time.

During the research periods I have attended three one-hour long PhD students' presentation and discussion sessions on research topics closely related to mine in terms of mass spectrometry technique. Although I initially felt nervous and intimidated attending, as most of the participants were PhD students or professors, later through my supervisor's encouragement I became very motivated to take the chance and connect with people to learn from their expertise. Through attending the meetings, I acquired valuable skills on how to interpret research data and present findings clearly and academically. This also gave me a chance to mindfully observe each PhD student's presentation methods, engagement technique, and time management skill, which guided me to reflect on myself from a different angle and identify areas for improvement that I can work on for future academic or non-

academic presentation opportunities. Moreover, the Q & A sessions during the meetings taught me the importance of being open and honest with myself in terms of knowledge gaps and helped me appreciate the value of other people's perspectives, particularly in academic settings, as they play a key role in shaping research to achieve more robust outcomes.

The impact the research has had on me

My time in this research significantly shaped my view on what practical academic research could possibly look like in industries or laboratory settings. Prior to starting the research, although I felt worried knowing it demands a high level of technical skill and knowledge, I never considered what it takes emotionally and mentally. Planning experiments, executing, and getting satisfactory results in each period was challenging, as experiments don't always yield expected results, either due to minor technical mistakes or unpredictable biological behaviour. This may have led me to unprecedented frustration, demotivation, and inconsistency, which could affect the research progress massively. However, during this time I learnt to sit with patience, repeat, and trust the process even when the results were unexpected.

Furthermore, this experience made me deeply aware of the impact I can have in the research environment and overall research success through applying my soft skills. As part of my research, I collaborated with two other fellow researchers, which gave an opportunity not only to share my perspective and enhance my communication skills, but also to refine my communication style to better engage with others. Through this refinement process, I developed an understanding of how cultural awareness, adaptability, and emotional intelligence play a role in shaping meaningful communication. Learning this will significantly contribute to my success in my future academic journey, research opportunities, and broader social endeavour.

Skill development

Skill development was one of the major personal growths I have made because of this research. The hands-on lab experience provided me with an opportunity to improve my technical skills and confidence in lab work, particularly, in sample preparation, sample handling and experimental protocols. I have developed new and very valuable skills in troubleshooting, handling and running a native mass spectrometer, enabling me to grow more into an analytical thinker through sharpening my insights in making meaningful interpretations for complex data and become an independent researcher in terms of designing experiments for my own research question in the future. Moreover, as these skills perfectly align with my academic discipline, it will meaningfully contribute to the success of my future.

This experience enhanced my reflection skill like never before, as I began to journal my day-to-day accomplishments in terms of new skills learnt and areas of improvement to evaluate daily efficiency, so that I plan for future action accordingly. This journaling allowed me to critically think back on each progress, analyse them, and identify my strengths and weaknesses, ultimately developing self-awareness. Additionally, journaling not only taught me tracking my progress but helped me work on my problem-solving skills through evaluating my setbacks, alternative approaches and executing effective solutions for better overall results.

Furthermore, my data interpretation and data analysis skills improved significantly because of this experience through learning new techniques, new software (i.e. Mass lynx and Deuters) and performing multiple data analyses from different experimental protocols under a single hypothesis. This strengthened my ability to integrate complex findings and present it in a scholarly standard. Alongside this I have remained open-minded, actively engaging with insights from fellow researchers and feedback from my supervisor. This has significantly contributed to the enhancement of my teamwork skills by fostering mutual respect and effective communication.

Future Plans

This summer research project gave me greater clarity about my interests and the career paths I wish to pursue. Although I enjoy doing research, I was previously unsure about my career paths and interests as a profession. However, this opportunity made me realise that I not only enjoy but also have a deep professional interest in cutting-edge research that can transform lives and advance healthcare systems, especially in fields such as neurodegenerative, cancer, and autoimmune disease research.

Furthermore, prior of this opportunity, I haven't had a plan for taking an industrial year placement as part of my degree, but inspired by this research, I have decided to take research-based placement year opportunities to further advance my research skills and broaden my experience to prepare myself for future career in research and the ability to undertake self-directed research.

Conclusion

My research period and other activities undertaken, such as residential programs and networking opportunities this summer as part of the Laidlaw program, have made a significant contribution to both my academic and personal growth in terms of skill development and strengthening global networks. Each experience has shaped and sharpened me through teaching me the values in becoming an ethical leader and using my passion in making a meaningful impact. Throughout this journey, I have learnt to remain open-minded to learn from

the experience of others, developing essential skills in problem solving, communication and critical thinking, which have enhanced my ability to lead effectively. Additionally, it taught me to appreciate collaboration in academic and non-academic settings as it contributes to the efficiency, effectiveness, and success of the project through providing new insights and fostering a sense of belonging, as it created an inclusive and supportive environment. This experience motivated me to pursue further research opportunities that challenge me to grow as a leader and contribute significantly.

Acknowledgements

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